From **DEPARTMENT OF NEUROBIOLOGY, CARE SCIENCE AND SOCIETY**

Karolinska Institutet, Stockholm, Sweden

INFLAMMATION AS A THERAPEUTIC TARGET FOR ALZHEIMER'S DISEASE

ERIK HJORTH

Stockholm 2010

Published by Karolinska Institutet. Printed by [name of printer]

The author may be contacted on erik.hjorth@ki.se

ISBN 978-91-7409-883-9

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which is characterised by impairment of memory and learning. The impairment is caused by neuronal death which originates in the parts of the brain that execute memory functions: the entorhinal cortex and hippocampus. The neuronal death is believed to be caused by the amyloid-β (Aβ) peptide which is prone to oligomerisation and aggregation into insoluble amyloid plaques (AP). The levels of soluble Aβ and the number of AP:s are increased in the AD brain which is attributed to increased production and impaired clearance of Aβ.

Another hallmark of AD, after neuronal death and the increased presence of Aβ, is inflammation in the form of activated microglia and increased levels of inflammatory proteins in the brain. Inflammation in the CNS has been shown to increase the production of Aβ and to impair, and even kill, neurons. On the other hand, inflammation has been shown to increase the removal of pathogens, such as Aβ, from the brain by increasing the phagocytic acticity of microglia. Inflammation is also associated with an increased secretion of neurotrophic factors that can protect neurons. Somehow this clearance of Aβ is impaired in AD and the levels of neurotrophic factors are decreased.

The work on this thesis has been focused on the inflammatory component of AD and how it can be modulated into performing activities that are beneficial for neurons without evoking the harmful activities of inflammation. For this purpose a human microglial cell line, CHME3, was characterized with regard to interaction with Aβ and inflammatory responses. The CHME3 microglial cell line was used as a model of inflammation in the human brain. In this model, we tested the ability of the neuropeptide α-MSH to inhibit inflammation induced by $A\beta_{1-40}$ and cytokines, and found that α-MSH, on the contrary, increased secretion of the inflammatory cytokine interleukin (IL)-6. α-MSH was also found to protect human neuronal cells against necrotic stress. The possibility of stimulating microglia into phagocytosis of $A\beta_{1-42}$ and the concurrent responses was also investigated. We found that the omega-3 fatty acid DHA and the adjuvant protollin had a stimulatory effect on phagocytosis of $A\beta_{1-42}$. DHA also had an inhibitory effect on the secretion of several cytokines. Microglia that were phagocytic of $A\beta_{1-42}$ displayed increased expression of several inflammatory

markers compared to non-phagocytic cells. Thus, inducible nitric oxide synthase (iNOS) was expressed to a larger extent on phagocytic cells. Treament with DHA abolished this imbalance in iNOS expression, indicating a less harmful phenotype of phagocytic cells upon treatment with DHA. Furthermore, inflammation in the form of IFNγ stimulated phagocytosis of $A\beta_{1-42}$ while decreasing the secretion of the neurotrophin brain-derived neurotrophic factor and inducing microglial cell death. $A\beta_{1-42}$ was also found to decrease the secretion of BDNF. The effect of omega-3 supplement on the levels of $A\beta_{1-42}$, tau and inflammatory markers in the cerebrospinal fluid and plasma of AD-patients was investigated in an intervention study. There were no detectable differences in any of the markers after 6 months of treatment with omega-3 compared with placebo control. However, at base line, a significant positive correlation between the soluble interleukin (IL)-1 receptor type II was observed.
The major finding in this thesis is the reduction in BDNF induced by $A\beta_{1-42}$, and the AD-related cytokine IFNγ, suggesting a new pathogenic mechanism in AD. Other important findings are the beneficial effect of DHA on microglial activities, which support the use of omega-3 supplements for treatment of AD. However, the results from the intervention study did not provide additional support for the use of omega-3, but suggest the importance of early intervention. α-MSH was found to promote neuronal survival suggesting a potential role for this peptide and its receptors for the treatment of neurodegenerative disorders.

LIST OF PUBLICATIONS

I. Lindberg C, **Hjorth E**, Post C, Winblad B, Schultzberg M
Cytokine production by human microglia: effects of β-amyloid and α-melanocyte stimulating hormone
Neurotoxicity Research, 2005, 8(3,4), 267-276

II. **Hjorth E**, Frenkel D, Weiner H, Schultzberg M
Effects of immunomodulatory substances on phagocytosis of $A\beta_{1-42}$ by human microglia
International Journal of Alzheimer's disease (accepted)

III. Freund-Levi Y, **Hjorth E**, Lindberg C, Cederholm T, Faxen-Irving G, Vedin I, Palmblad J, Wahlund L-O, Schultzberg M, Basun H, Eriksdotter Jönhagen M
Effects of Omega-3 Fatty Acids on Inflammatory Markers in Cerebrospinal Fluid and Plasma in Alzheimer's Disease: The OmegAD Study
Dement Geriatr Cogn Disord 2009;27:481–490

IV. **Hjorth E**, Vedin I, Palmblad J, Cederholm T, Freund-Levi Y, Faxen-Irving G, Wahlund L-O, Basun H, Eriksdotter Jönhagen M and Schultzberg M
Effects of omega-3 fatty acids on microglial phagocytosis and inflammatory phenotype
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid-β
AD	Alzheimer's Disease
α-MSH	α-Melanocyte Stimulating Hormone
AP	Amyloid Plaque
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CNS	Central Nervous System
COX	Cyclooxygenase
CSF	Cerebrospinal Fluid
DHA	Docosahexanoic Acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentanoic Acid
IL-(1β, 1Ra, 1RI, 2, 4, 5, 6, 8, 10, 12, 13)	Interleukin-(1β, 1Ra, 1RI, 2, 4, 5, 6, 8, 10, 12, 13)
IFNγ	Interferon-γ
LDH	Lactate Dehydrogenase
NF-κB	Nuclear factor-κB
NFT	Neurofibrillary Tangle
NMDA	N-Methyl-D-Aspartate
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAMP	Pathogen-Associated Molecular Pattern
PI3K	Phosphatidyl-Inositol-3-Kinase
PLA2	Phospholipase A2
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptor
RXR	Retinoid-X-Receptor
TNFα	Tumour Necrosis Factor-α

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

The connection between progressive cognitive decline and the presence in the brain of aggregates of the amyloid-β (Aβ)-peptide, and tangles of the protein tau, was first described by the German psychiatrist and neuropathologist Alois Alzheimer in a speech 1906. The syndrome was recognized as a distinct disorder which was given the name Alzheimer's disease (AD). The main pathological hallmark of AD is neurodegeneration, that is, the impairment and death of neurons.

Neurodegeneration in the central nervous system (CNS) is part of normal aging. In some cases this degeneration is accelerated as a manifestation of a pathological condition. This can be acute as in the case of head trauma or stroke. However, it can also be a slow progressive process as in the case of Parkinson's disease and the dementia-type diseases. The most common cause for dementia is AD which accounts for 50-70% of all cases [1]. With the increased life span associated with the modern world the prevalence of dementia is on the rise. In 2001 there were 24 million people in the world that were suffering from dementia and this number will double every year according to the prognosis [2]. The other major pathological hallmarks of AD beside neuronal loss are amyloid plaques (AP) and neurofibrillary tangles (NFT:s) [3; 4]. Although a great deal of controversy exists about the relative importance of AP:s *contra* NFT:s in AD, there is an overwhelming body of evidence showing that AP:s and the peptides they are composed of are culprits in the neurodegenerative processes in AD and based on this knowledge the "amyloid cascade hypothesis" has been formulated [5]. NFT:s are intracellular deposits of aggregated microtubule-associated proteins (MAP:s), and result from hyperphosphorylation of the MAP tau [6]. AP:s are composed of Aβ, which is secreted by neurons and other cells through cleavage of the larger, membrane-bound, amyloid-precursor protein (APP) [7; 8]. APP can be metabolised by two enzymatic pathways. One pathway is amyloidogenic, *i.e.* it results in the production of the aggregation-prone and neurotoxic Aβ. The other pathway is non-amyloidogenic and results in the production of a non-pathological peptide (P3) [9]. APP is metabolised by three enzymes: α-, β- and γ-secretase [10; 11]. Cleavage by α- and γ-secretase yields P3 while cleavage by β- and γ-secretase yields Aβ. In the metabolism of APP there is also production of an intracellular COOH-terminal fragment, β-amyloid precursor protein intracellular domain (AICD), which is believed to interact with the transcription of

genes [12]. Released together with Aβ or P3 are also the NH_2-terminal fragments, soluble APPα and β (sAPPα and sAPPβ). APP can be metabolized into Aβ peptides of different lengths. The 40 and 42 amino acid (aa) forms are believed to be the most produced variants, with the 42 aa form ($Aβ_{1-42}$) being the most abundant in plaques [13].

The Aβ peptides are prone to self-aggregation and deposition into insoluble plaques, but they also exist in the form of soluble monomers and oligomers, which are increased in the AD brain . Species of Aβ peptides and NFT:s have been shown to contribute to neuronal impairment and death in a number of studies, but the current view appears to emphasise the interaction between Aβ and NFT:s in the progression of the disease [14; 15; 16; 17; 18]. Aβ peptides have been shown to impair and kill neurons in a plethora of studies [19; 20; 21; 22; 23; 24; 25]. In the brain of a person suffering from AD there are higher levels of soluble Aβ-species and AP:s than in the non-AD brain [5]. This over-abundance has been attributed to increased production of APP, increased processing of APP by β-secretase and impaired clearance of Aβ [26; 27]. Somehow, the balance between production and clearance/degradation of Aβ is disturbed in AD. The fact that an inflammatory component exists in AD is well established. Increased levels of pro-inflammatory cytokines, such as interleukin (IL)-1 and IL-6, have been found in the brains of AD-patients [28; 29; 30], and there is an increase in the number of activated microglia and astrocytes in the areas of neurodegeneration and AP:s [31; 32]. Furthermore, elevated levels of inflammatory cytokines have been measured in serum and cerebrospinal fluid (CSF) from patients with AD [33; 34].

The clinical progression of AD is such that the cognitive disturbances are first observed as a decline in memory. Difficulties in storing and retrieving new information are the first symptoms to surface [35]. As the disease progresses there is a more general impairment in cognition and psychiatric symptoms such as apathy and depression may appear [36]. Finally, the cognitive decline is such that daily activities become impossible and at this stage the disease is associated with aggression, delusion and hallucinations. The clinical picture is mirrored by the pathological progression of the disease. Increased levels of AP:s and NFT:s, together with neuronal loss, are first seen in the entorhinal cortex and later progress to the hippocampus [37]. These areas are fundamental for the formation and retrieval of new memories [38]. Later in the disease there is a progression of the pathology to the rest of the cerebral cortex, which explains the general cognitive decline in the final stages of the disease [35; 39]. Parallel to the cortical degeneration

there is impairment and death of neurons in the basal forebrain [40; 41]. These neurons are cholinergic and project to the cortex and hippocampus in particular [42; 43]. The cholinergic input to the hippocampus is believed to synchronize and control the activity of the hippocampal neurons, thus making storage and retrieval of memory possible [44]. It is not known if disturbances in one area may worsen, or even cause, the problems in other areas but detrimental network effects are very likely.

1.2 THE IMMUNE SYSTEM AND INFLAMMATION

When the tissue of an organism is damaged by infection or trauma, its ability to cope with the challenges of the world, and to fulfill the demands of biological drives and instincts are reduced to a certain degree. In other words, homeostasis of the organism is lost. It is not hard to imagine that the evolutionary drive to develop toward restoration of homeostasis must have been very strong, and thus the immune system developed. The ability of single-cell organisms to engulf, phagocytose, threatening objects or organisms can perhaps be described as the first, archaic, form of immune defence. There is an appealing and immediate logic in the removal and destruction of the pathogenic entity. However, different forms of pathogens induced differentiation of the immune system so that other forms of immune responses evolved, producing an arsenal including free radicals, membrane-perforating molecules, apoptosis-inducing signalling, and antibodies. However, these responses may destroy an invading pathogen, but would do little for the restoration of tissue homeostasis and could in fact increase the level of tissue damage. To restore homeostasis the immune response must reach an endpoint where it is down-regulated, resolved, and rebuilding and healing of the tissue takes place [45]. The immune system promotes rebuilding and healing by inducing the secretion of growth factors that increase the survival of cells, stimulate them to regain their lost or impaired functions and increase their proliferation to fill the empty space [46; 47; 48].

The immune system has traditionally been divided into an innate and adaptive, or humoral, part. The innate system, which controls the response of inflammation, is the oldest part and is created with a kind of "general" understanding of what a foreign pathogen or debris is. The innate immune response is thus quite crude and can be harsh for the surrounding tissue. It is, however, on constant alert with cells placed in the tissues for the detection of disturbances, and can mount a quick response.

The adaptive part of the immune system is based on the production of antibodies, which recognise pathogens as something that does not belong to the organism. The antibodies are

secreted from B-cells which produce one type of antibody. By the interaction of B-cells with pathogens (or vaccines) the antibodies become more specialised, and the cells that produce the antibody in question become more numerous and responsive, in short the system adapts to the new situation. The marking of a pathogen with antibodies generally does not produce any effect on the pathogen, but guides immune cells to phagocytose the object, or produce lysis of the pathogen by the binding of complement proteins. The time frame of the adaptive immune response depends on if the immune system has encountered the pathogen before or is naïve. It is, however, regarded as much slower than the innate system, since it depends on the interaction with the innate system and if naïve, migration of cells in the circulation and lymphatic system.

The innate immune response can be divided into the complement system and the inflammatory response. The complement system, which is considered the oldest part of the immune system of multicellular organisms, is based on the assembly of proteins in a cascade forming a pore complex that produces lysis of the pathogen onto which it binds. The binding can be guided by interaction with the adaptive immune system through binding of antibodies, or by the recognition of pathogens by a protein in the complement cascade. Inflammation is induced by the recognition of a pathogen or foreign object by resident immunocompetent cells. Through a cascade of inflammatory signals there is recruitment of blood-borne immune cells into the tissue through the blood vessel wall, which is made permeable to cells by the inflammatory signals.

The immune response following a pathogenic or traumatic challenge can be illustrated as follows: A disturbance is induced in the tissue due to damage, or a cellular or molecular pathogen. The disturbance is detected by the innate immune system in the form of:

- debris from dead cells and destroyed tissue
- extracellular microorganisms in the form of associated molecular patterns
- intracellular microorganisms in the form of the presentation of abnormal proteins
- other immunomodulatory molecular entities that the immune system reacts to in general, and specifically, mediators produced from other cells for the coordination of the immune response

The first cells to encounter and respond to a disturbance in the tissue are resident macrophages and dendritic cells, both of which are derived from monocyte precursor cells [49; 50].

Monocytes can differentiate into different forms of macrophages depending on the microenvironment in the tissue that they enter, exemplified by the microglia in the brain or the Kupffer cells in the liver [51]. Macrophage-like cells such as microglia express a wide array of receptors with which the cells surveil the environment for disturbances [52; 53; 54; 55; 56]. The profile of receptors that macrophage-like cells express differs according to the activation phenotype, which in turn is determined by the signals that the cell receives [57; 58; 59; 60; 61]. If an object is recognized by one of these receptors, it will result in activation of the macrophage-like cell. Activation of the cell will result in changes in the behaviour of the cell. The pattern of secretion is changed, with increased secretion of immunomodulatory and effector molecules, such as free radicals. The consequence is that macrophage-like cells can be more or less responsive, or qualitatively different in their response to a specific disturbance as a result of the concurrent immunomodulatory signals that the cell receives.

Some of the immunomodulatory molecules that are secreted attract and activates blood-borne immune cells as well as resident cells, such as the endothelium so that adhesion molecules are expressed on the lumen side. This enables the blood-borne immune cells to attach to the vessel wall at the site of infection or trauma due to reciprocal expression of adhesion factors on cells and endothelium. Furthermore, the vessel wall becomes leaky upon activation of endothel, which permits tissue entry, or extravasation, of the activated cells adherent to the vessel wall. Another consequence of this is the flow of fluid into the tissue, which creates an oedema. The first cells to extravasate and execute their functions are the neutrophilic granulocytes , together with, or followed by, monocytes. As mentioned above, macrophages and microglia recognise pathogens by means of receptors, which are activated by molecular patterns associated with pathogens. This type of receptors are known as pathogen-associated molecular pattern (PAMP)-receptors, the most well-known being the Toll-like receptors (TLR:s), which recognize bacterial capsule components, among others. When a pathogen is recognized by a PAMP-receptor it activates the cell, with subsequent production of immunomodulatory molecules that coordinate the response of the immune system by endo-, para- and autocrine signalling. Activation of a PAMP-receptor may also induce a phagocytic response whereupon the macrophage will attempt to engulf the antigenic object. Phagcytosed antigenic material is normally degraded and presented on major histocompability complex type II (MHCII) proteins.

Neutrophils are actively phagocytic in the early phases of the immune response, while macrophages become more phagocytic in the later phases. Activated neutrophils also release

antimicrobial and immune signalling molecules, as well as secrete enzymes that degrade the extracellular matrix. The neutrophils die by apoptosis after performing their activities and are a major part of the pus that is formed at a site of lesion. After the neutrophils die, the macrophages clear the site of the apoptotic neutrophils, and the debris and pus that is the result of the rather harsh effort of the neutrophils. In the process of phagocytosis of the neutrophils the macrophages receives down-regulating signals that inactivate the inflammatory responses of the macrophages, which promotes the resolution of the inflammation, *i.e.* the phase where tissue rebuilding and healing can take place [62]. Recent research has also implicated soluble mediators in the induction of resolution [63; 64; 65; 66; 67]. In situations of chronic inflammation the resolution phase is not reached because of continuous inflammatory stimulation or lack of pro-resolving stimulation.

However, there is a parallel series of events that takes place at the same time and that determines the nature of the following responses. Shortly after the neutrophils enter the site, T-cells extravasate and are introduced to the pathogen by the interaction of MHC type I and II (MHCI and MHCII) and the corresponding T-cell receptor (TCR). CD8-positive T-cells are known as killer T-cells (T_K-cell) because of their cytotoxic activities, while CD4-positive cells are known as helper T-cells (T_H-cell) due to their function as coordinators of the immune response. The peptides presented on MHCI are derived from the metabolism of the presenting cell itself, and the purpose is to detect cells that have disturbances in their metabolism, that can be caused by intracellular pathogens or transformation. MHCI binds to CD8+ TCR:s and if the peptide is recognized as foreign the CD8+ T-cell will be activated and become a full-fledged T_K-cell that releases cytotoxic molecules to destroy the cell it is interacting with. The peptides presented on MHCII are derived from pathogens that were phagocytosed by macrophage-like cells or dendritic cells, and interact with TCR:s on CD4+ T-cells. If recognized by the naïve T-cell as foreign it will become an activated T_H-cell. AT_H-cell can then differentiate into subtypes of helper cells that secrete different molecules, thus directing the immune response into different response patterns. The choice of subtype is determined by the types of co-stimulation that the T_H-cell receives when its TCR is stimulated by the antigenic peptide. It is believed that the co-stimulation reflects the type of pathogen and that the T_H-cell differentiation is directed toward a subtype that can direct the immune response to the most effective response. There are four generally accepted subtypes of T_H-cells: T_{H1}-, T_{H2}, T_{reg} and T_{H17}-cells. T_{H1}- and T_{H2}-cells are considered the most important, since their role in making the decision of the immune

response to be dominated by an innate, cell-mediated (T_{H1}) or an adaptive, antibody-mediated (T_{H2}) profile.

1.2.1 Molecular mediators of the immune response

To activate cells of the immune system and guide them to the site of insult a system of communication is needed. This is accomplished by the secretion of molecules from cells activated by their detection of a tissue insult, and the expression of receptors for the same molecules on cells of the immune system. Receptors for the molecular mediators are not only expressed on immune cells, but also commonly on bystander cells. In many cases, the mediators have other effects apart from regulation of the immune system, such as the induction of apoptosis. Prolonged immune activation can therefore become pathological and have detrimental effects on the tissue. Mediators can be membrane-bound, thus requiring cell-cell contact, but the majority of immune mediators are secreted molecules.

1.2.1.1 Cytokines

The group of immune mediators that perhaps is most well-known is the cytokines. Cytokines are peptides or proteins that are produced not only by cells of the immune system, but also by other cell types. Cytokines have roles and functions beyond the regulation of the immune response, for example the pro-inflammatory cytokine interleukin (IL)-1β has an important role in the regulation of memory [68].

The distinction of cytokines into two major classes is based on the T_{H1}/T_{H2} categories of immune responses. Cytokines that promote a T_{H1} immune response are called pro-inflammatory, since inflammation is the cornerstone of the T_{H1} response. The most well-known T_{H1} cytokines are IL-1β, interferon (IFN)γ, tumour necrosis factor (TNF)α and IL-12. T_{H2}-inducing cytokines are called anti-inflammatory, since they inhibit inflammatory functions as well as promoting a humoral, adaptive immune response. The most well-known T_{H2} cytokines are IL-4, IL-10 and IL-13. Cytokines are synthesized and secreted "on-demand". The receptors for cytokines are expressed at low levels in the resting state, but the levels can increase drastically upon activation. Furthermore, there are several examples of decoy cytokine receptors and soluble cytokine receptors, which may or may not have signal transducing properties. Cytokines are very potent even at low concentrations, and can activate cells with a very low number of receptors. Stimulation with cytokines usually results in the secretion of even more cytokines and increased expression of cytokine receptors in a cascade. The effects of cytokines

are highly redundant and animals deficient in the gene for a particular cytokine can appear normal and healthy.

1.2.1.2 Chemokines

Chemokines, or chemotactic cytokines, are also peptide or protein mediators, and they are also synthesized and secreted on-demand upon activation. Chemokines act as a homing signal, chemoattractant, for immune cells and are important for assembling the immune cells at the site of insult, and they have been implicated in the pathology in AD [69]. Thus, cells at the site of insult become activated and secrete chemokines. A chemokine concentration gradient appears in the space around the secreting cells, and cells that express the receptor for the chemokine respond by moving in the direction of the gradient.

1.2.1.3 Arachidonic acid derivatives

Another type of immune-regulating messenger is the fatty acid derivatives, eicosanoids, that are produced from the phospholipids in the cell membrane. Phospholipase A2 cleaves arachidonic acid (AA) from the membrane and AA can then take part in several biosynthetic pathways that can result in the formation pro- and anti-inflammatory lipid mediators, although production of anti-inflammatory lipids downstream of PLA2 has been reported [70]. Metabolism of AA by cyclooxygenase (COX) creates prostaglandins, prostacyclins and thromboxanes, depending on which enzyme that comes downstream of COX [71], while metabolism of AA by lipoxygenase creates the members of the leukotriene family of eicosanoids .

1.2.1.4 Cell-cell contact

An important type of signalling involved in the regulation of the immune response is the ligation of two membrane-bound molecules on different cells. This type of signalling is dependent on the close proximity of the transmitting and responding cell. The interaction between an antigen-presenting cell and a T_H-cell *via* MHCII and TCR is an example of this kind of signalling. Another important example is the signalling between macrophage/microglia-bound CD40 and T-cell/astrocyte-bound CD40L, resulting in an inflammatory activation [72; 73].

1.2.2 Intracellular signalling and gene activation in inflammation

For the soluble and membrane-bound mediators to exert their effects after binding to their receptors, a system of intracellular signalling transmits the message to the effector molecules

and genes. The most important among these is the nuclear factor (NF)-κB pathway, which has become almost synonymous with inflammation. NF-κB is a complex of two proteins: a transcription factor, which is the active NF-κB, and an inhibitor called IκB. Inflammatory stimulation, such as IL-1β and TNFα, activates IκB kinase (IKK), which phosphorylates IκB, causing its release, resulting in a now active NF-κB, which translocates to the nucleus where it activates inflammatory genes [73; 74; 75; 76; 77]. Another pathway that is activated by cytokines is the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which is induced by, among others, the pro-inflammatory cytokines IFNγ and IL-6, and which mediates inflammation by activation of genes displaying a STAT-motif [78; 79]. Many studies have shown that the NF-κB and JAK/STAT pathways interact to produce the inflammatory response [80; 81; 82]. There is also an increased activation of the mitogen-activated protein kinases (MAPK) in activated cells, which contributes to the activation by enhancing secretion, proliferation and morphological changes. In particular, the p38 and c-jun N-terminal kinases (JNK) have been implicated in inflammation. JNK is also an activator of the transcription factor activator protein (AP)-1 which regulates several important inflammatory genes. Signalling mediated by increased levels of cAMP has been shown to inhibit inflammation, but it may be stimulatory under some circumstances. Signalling through the phosphatidylinositol-3-kinase (PI3K) pathway is another pathway shown to activate NF-κB. A pathway that appears to have both pro- and anti-inflammatory forks is the peroxisome-proliferator associated receptor (PPAR) pathway. PPAR proteins bind fatty acids, such as eicosanoids, and thereafter regulates a number of target genes by binding to and activating the retinoid-X-receptor (RXR) [83; 84; 85].

1.3 INFLAMMATION IN THE CENTRAL NERVOUS SYSTEM

The CNS is different from other tissues in the body. It is shielded from the rest of the organism by the blood brain barrier (BBB), which controls the flux of cells and molecules into (and from) the CNS. Under normal conditions the BBB should be closed for circulating cells of the immune system, and if they are present in the CNS, it is a sign of a traumatic or pathological event. An exception is the influx of monocytes that is believed to replenish the population of resident microglia under normal conditions. The demanding and complex type of work performed by neurons is sensitive to disturbances. If immune cells, with their potentially harmful activities, had unlimited access to the brain the consequences could be dire. It is clear that the brain, being at the top of the hierarchy of the body, is presented with unique challenges

when it comes to maintaining homeostasis. In the normal CNS, the immune system is represented by the microglia. However, astrocytes can perform many functions associated with the immune system, such as cytokine production.

1.3.1 Damage and impairment of neurons caused by inflammation

1.3.1.1 Oxidative stress

Free radicals generated during inflammatory activation can cause damage to neurons by destroying macromolecules. Neurons are highly dependent on the integrity and fluidity of their cell membrane to execute their functions effectively. Lipids in the cell membrane are highly susceptible to lipid peroxidation by free radicals. The lipid peroxidation also produces aldehydes that can bind to, and destroy, macromolecules vital to the cell [86].

1.3.1.2 Pro-apoptotic stimulation

The pro-inflammatory cytokine TNFα has been shown to induce apoptosis in neurons [87]. The receptor for TNFα has a moiety in its intracellular signalling domain called a death domain (DD). DD:s exist on other members of the TNFR-family, on receptors for other cytokines such as the IL-1 receptor type I (IL-1RI), and on PAMP-receptors, although the evidence for a pro-apoptotic effect is less extensive than for TNFα. The mere existence of a DD does not imply that ligand-binding results in apoptosis. The decision of dying, or not, is taken with regard to the total pro- and anti-apoptotic signals that are being transduced into a cell at any given time.

1.3.1.3 Negative effects on neuronal plasticity

The pro-inflammatory cytokines TNFα and IL-1β has been shown to raise the threshold for the induction of long-term potentiation (LTP), which is a major mechanism for the formation of memories. Inflammation can thus directly impair learning and cognition [88; 89; 90].

1.3.1.4 Excitotoxicity

Inflammation has been shown to decrease the expression of glutamate transporters on astrocytes. These transporters remove glutamate from the synaptic cleft, secreted during neuronal signalling. If glutamate remains in the synapse in high concentration the post-synaptic cell may be overstimulated. The net result of overstimulation of glutamate receptors is an increase in the intracellular levels of Ca^{2+}, which creates a type of toxicity called excitotoxicity [91; 92]. Calcium ions are important intracellular messengers that regulate enzymes that, if left

active and without control, can induce apoptosis or make the cell go into necrosis directly. It has also been shown that quinolinic acid, which is a glutamate receptor agonist, is released from activated microglia and may contribute to excitotoxicity [93; 94; 95].

1.3.2 Cellular mediators of inflammation in the CNS

1.3.2.1 Resident cells – microglia, astrocytes and neurons

In the CNS, several cell types can contribute to the inflammatory response. Microglial cells have long been considered the main inflammatory cells in the CNS. In the healthy CNS, microglia are in a resting state with ramified processes that are surveilling the environment for disturbances. When activated, the microglia proliferate and change morphology into a round amoeboid shape that is similar to that of activated macrophages. Activation also increases the secretion of inflammatory mediators and the expression of their receptors. Another type of cells that can induce and modulate inflammation in the CNS is the astrocyte. Astrocytes are derived from neural precursor cells, not from bone-marrow derived mesenchymal stem cell as proper immune cells (and microglia). Astrocytes have some similarities to T_H-cells, such as production of IFNγ and expression of CD40L. They can produce reactive oxygen and nitrogen species and perform phagocytosis [96; 97]. The main task of astrocytes, however, appears to be the maintenance of neurons and their functions. An important task of astrocytes is the clearance of neurotransmitters from the synaptic cleft which prevents neuronal overstimulation as described above. Astrocytes are also constituents of the BBB. Confronted with an insult, astrocytes and microglia interact to produce an inflammatory response. This response can differ in secretory products and other activities according to the type of stimulation induced by the insult. It is also known that neurons and microglia interact to modulate the inflammatory response. For example, neuronal activity induces the release of fractalkine from the neuron, and this has an inhibitory effect on harmful microglial activities [98; 99]. Another example is the secretion of the neurotrophin brain-derived neurotrophic factor (BDNF) from active neurons, which has been shown to modulate microglial activation [100; 101].

Cells migrating from the periphery

Monocytes have been shown to cross the BBB with subsequent release of inflammatory factors. The distinction between monocytes and microglia is blurred, since it is believed that microglia are monocytes that have entered the CNS and differentiated into microglia due to the microenvironment of the brain parenchyme. The contribution of microglia *vs* monocytes

to the harmful and beneficial responses associated with inflammation in the CNS is unclear, mainly due to the difficulties in distinguishing the two cell types from each other. While resting microglia have a morphology that is different from unactivated monocytes, the morphology of activated microglia is similar to that of activated monocytes, macrophages, and they also have a similar expression of cellular markers. The notion that microglia are derived from monocytes, together with the large overlap in responses and characteristics in activated microglia and monocytes, raises the question if the distinction is meaningful.

1.4 MICROGLIAL ACTIVITIES IN INFLAMMATION

1.4.1 Secretory products

1.4.1.1 Cytokines

Just like other macrophage-like cells, microglia are capable of synthesizing and secreting a wide range of pro- and anti-inflammatory cytokines. The cytokines that have been most studied with regard to microglial secretion are IL-1β, TNFα and IL-6.

1.4.1.2 Growth factors

Microglia can also secrete growth factors that can stimulate healing and regrowth of the tissue. Of great importance for AD is the secretion of BDNF. Many studies, published during the last 30 years demonstrate the importance of BDNF in memory formation and neuroprotection [102; 103; 104; 105; 106; 107; 108; 109]. Nerve growth factor (NGF) is another neuroprotective growth factor that microglia can produce [110]. The growth factors secreted by microglia can also act in an auto- or paracrine manner. As was discussed above, BDNF is secreted from microglia and has been shown to modulate activation of microglia.

1.4.1.3 Oxidative stress

Microglia can be a source of oxidative stress through the induction of inducible nitric oxide synthase (iNOS). Free radicals are also produced by cyclooxygenase (COX) enzymes during the production of eicosanoids. Another source of oxidative stress is the lysosomal enzymes, which are active in lysosomal degradation of phagocytosed debris or pathogens.

1.4.2 Phagocytosis and degradation of debris and pathogens

For the body to be cleared of debris from tissue turnover, pathogens and foreign objects, a system for removal, clearance and degradation must be in place. Removal of objects from the tissue is carried out by the process of phagocytosis. Phagocytosis is mainly performed by cells

of the immune system. Particularly, macrophage-like cells, dendritic cells and neutrophils are proficient at phagocytosis.

1.4.2.1 The mechanism of phagocytosis

For phagocytosis to take place, the cell must recognize an object as being "worthy" of phagocytosis. As described above, an array of surface receptors fulfill this role by acting as sensors on objects encountered in the tissue. If the object is recognized as antigenic, a signal is transduced into the cell and a rearrangement of the cytoskeleton takes place to engulf the object. When completely engulfed, the object is in an intracellular vesicle called phagosome. The recognition of the object by the receptor can have other consequences than engulfment. Many phagocytic receptors can also mediate an inflammatory activation of the cell. An exception is the CD18 molecule that recognizes the phoshatidylserine moieties on the outer leaflet of the membrane of apoptotic cells without activation. Apoptotic cells are ubiquitous in most tissues due to normal cell turnover. It would cause unnecessary damage and stress if an inflammation was induced everytime a cell dies. As mentioned above, the PAMP-receptors can mediate phagocytosis. Another type of receptor that can mediate phagocytosis is the scavenger receptors. The scavenger receptors recognize, for example, oxidized peptides and stimulate their uptake. Fc-receptors can mediate phagocytosis of objects that have been opsonized with antibodies. Complement fragment 5a binds to bacterial surfaces and stimulates their phagocytosis when binding to complement receptors on microglia or macrophages.

1.4.2.2 The mechanisms of degradation

The invagination produced by the phagocytic process will close and become the phagosome, an intracellular vesicle that contains the phagocytosed material. The phagosome later docks and fuses with a lysosome, forming a phagolysosome. It is in the phagolysosome that degradation of the phagocytosed material takes place. The milieu in a phagolysosome is acidic, which promotes denaturation. Oxygen-independent degradation occurs by proteases, lipases, amylases and nucleases in the phagolysosome, digesting the phagocytosed material. Oxygen-dependent degradation is performed by activation of a respiratory burst. The respiratory burst is the production of oxidative stress by NADPH oxidase and myeloperoxidase, which are meant to destroy bacteria in the phagolysosome. This oxidative stress can have detrimental consequences for the surrounding tissue.

1.4.3 Inflammation and AD

The connection between AD and inflammation may be stronger than just activation of microglia or other cells induced by the neuronal cell death in the AD-brain. Aβ has been shown to have pro-inflammatory effects on microglia, as well as astrocytes, in several studies [111; 112; 113; 114]. Inflammation has been shown to increase the production of APP, and to increase amyloidogenic metabolism of APP [115; 116; 117]. Theoretically, this is suggestive of a vicious circle which starts with an increase in either the levels of Aβ or inflammatory mediators, and which later becomes self-sustaining. Another question that is raised is why the activated microglia and astrocytes are unable to remove the excessive levels of Aβ from the brain by phagocytosis. Inflammation increases phagocytosis, but with regard to the AD brain, it appears that phagocytosis is either insufficient or inhibited. Some of the ways in which inflammation can contribute to the pathology in AD are summarized in Fig. 1.

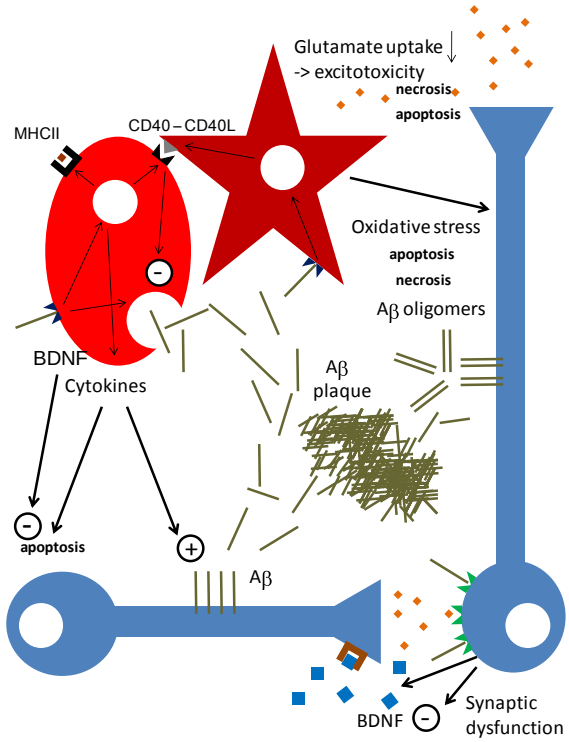

Fig. 1. Pathological interplay between Aβ and inflammation in AD. The major neurotoxic pathways of Aβ according to the author are presented in this figure: (a) Oligomeric Aβ can induce toxicity directly on neurons. (b) Aβ induces synaptic dysfunction with potential disturbances in neuronal BDNF secretion. (c) Activation of microglia and astrocytes by Aβ increases oxidative stress and secretion of inflammatory cytokines. (d) Binding of CD40 – CD40L, which are increased by Aβ, further activates the microglia and inhibits phagocytosis of Aβ. (e) impaired glial uptake of glutamate may cause excitotoxicity.

1.5 MODULATION OF THE INFLAMMATORY RESPONSE INTO A THERAPY FOR AD

The immune system is capable of a wide range of responses that can be destructive or protective for neurons. The main strategy would be to invoke an immune response that stimulates beneficial activities, but at the same time inhibits activities that can be detrimental. To remove a disease-causing pathogen is in most cases probably the most effective way of treating a disease. In the case of AD, the main pathogen is Aβ. To stimulate cellular uptake, phagocytosis, of Aβ is a promising strategy for treatment for AD. In some animal studies, stimulation of phagocytosis by immunization have been shown to be effective in removing plaques and to promote cognitive performance [118]. Human clinical trials with passive immunization have been started, but were aborted due to serious side effects in a few participants, highlighting the danger of manipulation of immune responses: stimulation into phagocytosis can activate the cells into performing unwanted and damaging activities. The activities that should be inhibited are those associated with a full-blown inflammation such as secretion of pro-inflammatory molecules and production of oxidative stress. Growth factors secreted by microglia and astrocytes can protect and enhance the activities of neurons. Secretion of growth factors is a valuable therapeutic target in itself. Stimulation of glial or neuronal production of growth factors would be beneficial for many disorders in the CNS. When evaluating therapies for AD, the effects on microglial and astrocyte growth factor should be taken into account. The reciprocal stimulatory relation between inflammation and Aβ has been discussed in this thesis. It is conceivable that just decreasing the level of inflammation in the AD brain can help to break the vicious circle.

2 THESIS AIMS

The primary aim of this thesis is to develop a strategy for using inflammatory responses in the treatment of neurodegenerative conditions particularly with regard to AD, *i.e.* to investigate if the mechanisms of inflammation can be controlled in a way that harmful responses are down-regulatedm while beneficial responses are enhanced. Most studies on the influence of inflammation on neurodegenerative conditions that have been performed in the past, have focused on the global repression of inflammatory responses, *i.e*. positive outcomes of the experiments have been defined as the absence of activity of the immune cells studied. However, it may be that when repressing the inflammatory response, the beneficial activities that are associated with inflammation are also repressed. Inflammation is a process with an ending, resolution, which previously has been viewed as a more or less passive down-regulation of the inflammatory response due to the absence of activating stimuli. In recent studies, resolution has been suggested to be an active process that is mediated and controlled by signalling molecules and their receptors.

2.1 SPECIFIC AIMS

To realize the primary aim of this thesis the following three specific aims were formulated:

- to characterize human CHME3 microglia with regard to interactions with Aβ and inflammation, with special emphasis on phagocytosis of Aβ
- to investigate if immunomodulatory substances can stimulate phagocytosis of Ab and increase neuronal survival and function through direct effects on neuronal cells or indirect effects on microglia
- to investigate the effects of omega-3 on inflammatory factors in CSF and plasma from AD patients

3 METHODOLOGY

3.1 GENERAL OVERVIEW

A number of methods and techniques were utilized to perform the experiments and producing the data presented in the studies of this thesis. In this section we will describe the methods and techniques, discuss their benefits and drawbacks, and compare them to alternative methods used to analyze the same type of material.

3.1 CELLULAR MODELS – PAPERS I, II, IV AND PRELIMINARY RESULTS

3.1.1 A model of human microglia: The human CHME3 microglial cell line

The most appropriate *in vitro* model of human microglia would be to use human primary microglia from aged controls and AD-patients. However, the availability of human primary microglia is extremely limited for obvious reasons. Microglia derived from aged humans are only available from *post mortem* material or excess material from brain surgery. In addition, the methodologies of culturing adult human microglia, and the ethical and administrative issues, are non-trivial. An alternative source of human primary microglia is to isolate microglia from aborted human fetuses which is less demanding than work on adult microglia. However, there is still a problem with insufficient number of cells for the purpose of the experiment. Added is also the issue of extrapolating the responses of embryonic cells to aged cells. Transformed, immortalized, cell lines are commonly used in models of pathology to mimic their primary cell counterparts. The CHME3 microglia cell line used in this thesis was created by transfection with SV40 large T antigen [119]. Transformation caused by SV40 large T antigen immortalizes the cell by the expression of the gene product which binds to the p53 and retinoblastoma tumour suppressor protein [120]. The abolished anti-tumour defense results in transformation of the cells through spontaneous mutations that remain in continuous generations by removal of the function of proteins that detect genomic abnormalities and control cell-cycle progression. This allows for the continouos and fast proliferation of cells that normally would proliferate slowly and finally go into senescence. The drawback of using cell lines is that by removing parts of the system that controls the genomic integrity of the cell, it is possible that the cell line will change with time due to accumulated mutations, thus changing the original phenotype of the cell line. Another disadvantage is that the inhibition of the cellular control machinery may affect signalling pathways that are relevant for experimental results. There are no commercially available human microglial cell lines and to the author's knowledge, only two human cell lines

exist, with the CHME3 line being one of them. Fig. 2 shows the CHME3 microglial cells in culture

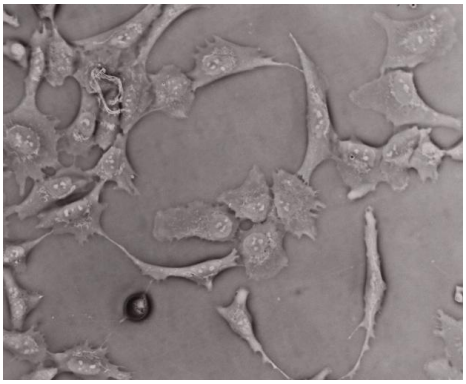

Fig. 2. This microphotograph, taken in an inverted microscope, shows human CHME3 microglia in culture. Note the morphological heterogeneity of the cells with both elongated, process-bearing cells and amoeboid cells.

3.1.2 A model for neurodegeneration on human neuronal cells

The human neuroblastoma SK-N-SH SY5Y (SY5Y) is one of the most commonly used models of human neurons. SY5Y cells express several neuronal proteins and activities, but they are highly mitotic and are morphologically different from primary neurons. A common method for neuronal differentiation of SY5Y cells is the treatment with retinoic acid [121]. This treatment increases the expression of neuronal proteins and induces changes in morphology toward a neuronal phenotype, *i.e*. neurite extension, although the cells are still mitotic. One neuronal protein that is upregulated by retinoic acid is TrkB, which is the receptor for BDNF [122]. If the SY5Y cells are first treated with retinoic acid, then treated with BDNF in serum-free medium for an extended period of time, neuronal morphology and biochemical profile is further enhanced, the cells become post-mitotic and projects an extensive neurite network.

3.1.3 Immunochemistry

Immunochemistry, or the use of antibodies to analyse different types of samples, is a versatile technique that is involved in several of the methods used in the papers of this thesis. **Immunocytochemistry** (ICC) is the use of antibodies to "stain" cells, which can then be analysed with microscopy or flow-cytometry. **ELISA** (enzyme-linked immunosorbent assay) is a similar method based on antibodies, and used to quantify molecules in solution. **MesoScale** multiplex analysis is based on the same principle as ELISA, but the detection system is different and the method allows simultaneous analysis of several molecules. The methodology used in the studies in this thesis is based on indirect immunofluorescence, *i.e.* secondary antibodies that

are reactive to the species producing the primary antibody are added after incubation with the primary antibody. The secondary antibodies are labelled with different types of markers for detection of the immunoreaction, and in our work we have used secondary antibodies labelled with fluorescent markers, hence immunofluorescence. The use of indirect immunofluorescence will produce an amplification of the signal from the molecule investigated, since several secondary antibodies can react with one primary antibody. The direct immunofluorescence from a primary antibody directly labelled with a fluorophore is usually too weak to be detected in the microscope. Directly labeled primary antibodies are commonly used in flow-cytometry but have the disadvantage of being expensive.

3.1.3.1 Visualization in the microscope

Inspection in the microscope was used for qualitative assessment of samples. Both regular fluorescent and confocal microscopy were utilized. Confocal microscopy uses laser to render a slice of immunofluorescene from a sample at a particular z-value. If a series of consecutive slices is rendered, a 3-dimensional image is created. By immunochemical staining for relevant markers, it is thus possible to assess the subcellular location of, for example, a phagocytosed object. Quantitative assessment in the microscope is possible, but time-consuming, and subject to bias.

3.1.3.2 Quantification of phagocytosis and immunoreactivity of cells with flow-cytometry

Cytometry is the technique of measuring characteristics of cells. In flow-cytometry the cells are measured as they flow past a detector, one by one, so that the characteristics of each individual cell are emitted as a signal. The signal is produced by projecting laser light on the cells, and the light that is reflected, or emitted, from the cells is detected by photo-detectors. A common application of flow-cytometry is to produce specific information about the presence of a certain molecule, condition or process in the cells. To accomplish this, the cells are incubated with fluorescent antibodies or dyes for the detection of a certain molecule, physical condition or process. Data from flow-cytometry are commonly presented as scatter plots in an analysis program. In these plots it is possible to establish borders that determine if a signal is positive or not, thus yielding binary quantitative data. The borders are established by analyzing the negative controls, which set the limit for the cellular presence of the molecule analysed.
To effectively produce unbiased, quantitative data of the cellular presence of markers or phagocytosed material, we developed a technique for analysing adherent cells with flow-cytometry. This technique was used to quantify phagocytosis, cellular markers and the data on

apoptosis as assessed with the TUNEL-assay presented in the Preliminary results. This method is used extensively for the analysis of blood-borne cells, which are non-adherent on cell plastic. In this thesis we analysed microglia, cells that are strongly adherent, which makes analysis complicated. Adherent cells must be detached mildly, not to affect the characteristics that are to be quantified. In addition, enzymatic detachment which is the most common method for detaching cells brings the risk of degrading epitopes of interest. To avoid this, the cells were detached with a commercial buffer containing salts and chelators that promote a mild form of detachment.

3.1.3.3 Outline of the experiments

Human CHME3 microglia and neuroblastoma SY5Y cells were routinely cultured and sub-cultured under sterile conditions. In all experiments the cells were seeded in standard multi-well plates or dishes. Before the treatments, the wells or dishes were washed with serum-free medium and left with this for 1 h, after which the treatments were added to the cultures. All treatments were prepared in serum-free medium. The importance of avoiding serum when performing experiments on immuno-competent cells is highlighted by the presence of immunoactive substances in serum that can influence the experiments. Cell proliferation and cell death assays were performed immediately at the end of the experiment. Cells destined for ICC and analysis in the microscope were dried on to their coverslip and fixed with 4% paraformaldehyde (PFA). Cells destined for analysis of phagocytosis and immunoreactivity were detached and the resulting cell suspension was fixed with 1% PFA. The lower concentration of PFA used for fixation of the cell suspension is motivated by the background fluorescence of PFA that can create problems in the analysis. However, we have found that PFA also promotes attachment of the cells to the coverslip and hence the higher concentration was used for microscopical analysis.

3.1.3.4 Analysis of phagocytosis

The intracellular presence of a labelled object can easily be detected and quantified with flow-cytometry or manual microscopical inspection (see above). However, evidence is needed to show that the object is phagocytosed and not attached to the cell in an unspecific manner. To confirm intracellular localisation and phagocytosis, ICC for lysosome-associated membrane protein-2 (lamp-2) was performed and analysed by confocal microscopy for the co-localization of $A\beta_{1\text{-}42}$ with lamp-2.

Flow-cytometry was used to quantify the phagocytosis of $A\beta_{1-42}$ or latex beads by the CHME3 microglia. First, control samples incubated without the fluorophore-labelled $A\beta_{1-42}$ or latex beads were analysed, generating a base line signal in the wavelength channel of the fluorophore. In subsequent analyses, all cells that emitted a signal that was higher than this base line were considered phagocytic, and phagocytic activity in a culture was expressed as the percentage of phagocytic cells.

3.1.3.5 Analysis of cellular phenotype

The phenotype of CHME3 microglia was analysed with flow-cytometry by quantification of cells being immunoreactive to the cellular markers investigated. A base line separating positive and negative cells was generated in analogy to the methodology on phagocytosis, and the immunoreactivity to the marker was expressed as a percentage of the total number of cells.
To determine the phenotype of phagocytic and non-phagocytic microglia, the differential expression of the cellular markers in the phagocytic and non-phagocytic populations were assessed. Immunoreactivity was expressed as the percentage of cells being positive for a marker in the phagocytic, or non-phagocytic, population. A difference in the percentage indicates a difference in phenotype.

3.1.3.6 Analysis of secretory products

The secreted levels of relevant molecules were analysed in all papers included in this thesis. With regard to this, it is important to note that the level of a secreted product is subject to several factors that influence the appreciated level of a molecule. Spontaneous or enzymatic degradation can influence the levels, as well as sticking of the molecules to cell membranes or culture dishes.

3.1.3.6.1 ELISA (enzyme-linked immunosorbent assay) and MesoScale multiplex analysis

ELISA is the most common method to analyse secretory products from cell cultures or bodily fluids, such as CSF or serum. It is a well-established and uncomplicated assay that can be optimized by the end user for special applications. The drawback is that there is a relatively high variability in this method, which is a consequence of the immunohemistry utilized, and the detection system, usually based on absorbance. The secondary (or detection) antibody is commonly conjugated with an enzyme that after addition of a substrate yields a product, the detected level of which is proportional to the concentration of the molecule investigated. In

some ELISA:s, fluorescence or luminescence replaces absorbance as a detection system, thus increasing sensitivity and reliability.
Another drawback of ELISA is the large amount of sample that is consumed, especially if several molecules are to be analysed in each sample. To overcome these limitations multiplexed analyses have been developed. In **paper IV**, the MesoScale multiplex technique was used for the measurement of a set of T_{H1} and T_{H2} cytokines. This technique does not only allow analysis of several antigens in a small sample volume, but is also a lot more sensitive than an absorbance based ELISA, since it is based on luminescence. Furthermore, with the MesoScale technique, the luminescence is activated by electricity which reduces the background problem.

3.1.3.7 Analysis of cell proliferation and cell death

Monitoring of the well-being of the cells is a routine task to help interprete other data, such as secretion. In studies on neurodegeneration, the data from proliferation and cell death assays are commonly the primary outcome of the experiment, as in the case of the Preliminary results included in this thesis.

3.1.3.7.1 The MTT-assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is an old 'workhorse' in the studies on cellular proliferation and death. It is well-established, and relatively uncomplicated and therefore still commonly used despite the surfacing of related, more sensitive, but more expensive assays. The assay relies on the activity of mitochondrial reductases, which are active in healthy, living cells. The MTT salt is a substrate to these enzymes and if added to living cells they will produce a coloured product, the absorbance of which is measured and proportional to the amount of living cells in the culture. Data from the MTT-assay are usually normalized to the control treatment data (= 100%).

3.1.3.7.2 The LDH-assay

The LDH (lactate dehydrogenase)-assay is also an old workhorse in studies on cellular toxicity for the study on necrosis. Necrotic cells cannot maintain their membrane integrity, resulting in leakage of cytosolic molecules, such as LDH, into the culture medium. Measurement of the LDH-activity in the culture medium gives information about the degree of necrosis in the culture. The LDH-activity is assessed by adding a substrate to a volume of conditioned medium and then measuring the absorbance of the product, that is proportional to the amount of necrosis

in the culture. The data from the LDH-assay is normalised to a positive control, *i.e.* cells lysed with a detergent, thus creating 100% cell death.

3.1.3.7.3 The TUNEL-assay

To analyse apoptosis, yet another established assay was used. The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling)-assay is performed on fixed cells in suspension or cells attached to a coverslip. In apoptosis there is a characteristic fragmentation of DNA, creating a large number of free endings that are recognised by the enzyme terminal deoxynucleotidyl transferase (Tdt), adding nucleotides to these endings. In the TUNEL-assay, the reaction is performed in the presence of fluorophore-conjugated nucleotides resulting in fluorescent labelling of apoptotic nuclei. The number of apoptotic nuclei is expressed as a percentage of the total number of nuclei. The quantification of apoptosis can be performed manually by counting positive nuclei in the microscope. However, the quantification is faster and more reliable by analysing a cell suspension in a flow-cytometer. The data on neuronal apoptosis in the Preliminary results were generated in this way.

3.2 EFFECTS OF INTERVENTION WITH EPAX ON INFLAMMATORY MARKERS IN AD-PATIENTS – A CLINICAL RANDOMIZED TRIAL

Paper III in this thesis is part of the OmegAD-study, which is aimed at investigating the potential of omega-3 fatty acids for the treatment of AD. In the OmegAD-study a global approach has been applied with regard to outcome variables. Cognitive function, and nutritional status were assessed, as well as the levels of inflammatory markers, which is the focus of **paper III** in this thesis.

3.2.1 The study population

In the original OmegAD-study, 361 patients were recruited from the specialist memory clinics at Karolinska University Hospital, Huddinge, and from Danderyd Hospital and St. Göran Hospital in Stockholm. The patients were included on the basis of the diagnosis of mild to moderate AD according to DSM-IV classification. All patients were medicated with acetylcholine esterase-inhibitors for at least 3 months before the start of the study and this treatment continued during the study. The population studied in **paper III** is a subgroup of 40 patients that were the 40 first participants included in the OmegAD study. Patients under medication with non-steroidal anti-inflammatory drugs (NSAIDS) were not included.

3.2.2 Study design

The participants were randomly assigned to EPAX (see 3.3.5) or placebo groups. At baseline the participants were subject to cognitive and neuropsychiatric evaluation as well as anthropometric assessment. Blood was sampled from all participants and CSF was sampled through lumbar puncture in a subset of the participants. Treatment with EPAX/placebo continued for 6 months, after which new evaluation and collection of blood and CSF was performed. After this all participants were given EPAX for another 6 months after which the last evaluations were performed.

The plasma and CSF were analysed with commercial ELISA for the levels of IL-6, TNFα and sIL-1RII. High sensitivity ELISA was used to analyse the levels of IL-6 and TNFα in CSF. CSF samples were sent to Clinical Neurochemistry Laboratory, Mölndals Lasarett, Gothenberg, Sweden, for analysis of t-tau, p-tau and $A\beta_{1-42}$. The plasma levels of high sensitive C-reactive peptide (hsCRP) was analysed at Dept. of clinical chemistry, Karolinska University Hospital, Stockholm, Sweden.

3.3 INTRODUCTION TO THE IMMUNOMODULATORY SUBSTANCES USED

To test the possibility of influencing the inflammatory response, a number of substances with immunomodulatory properties were tested in the human microglial model. Furthermore, to investigate the effect of an intervention with omega-3 fatty acids, AD patients were treated with EPAX (see 3.3.5), a mixture of omega-3 fatty acids. In the Preliminary results we present data from studies on the potential of α-MSH to protect human neuronal cells from necrosis and apoptosis.

3.3.1 Protollin

Protollin is an adjuvant made of hydrophobic outer membrane proteins from *N. meningitides* which are non-covalently complexed with LPS from *Shigella flexneri* 2a. Protollin has been shown to stimulate removal of Aβ from transgenic AD-mice [123], an effect that the authors attributed to microglial activation, through activation of TLR type 2 and 4. This substance cannot pass the BBB through normal routes, but nasal administration can be used for transport into the CNS.

3.3.2 **IL-1β**

Interleukin-1 is one of the two archetypical pro-inflammatory cytokines. It is produced by a number of cell types, although the secretion from cells of the monocyte lineage is the most studied. IL-1β is secreted, while its sibling IL-1α is mostly encountered in a membrane-bound form. They both activate the same receptor, IL-1R type I, which is membrane-bound. There is also a secreted form of this receptor, sIL-1R type I, which can act as a decoy receptor, but can also initiate signal transduction if its complex with IL-1 is inserted into the membrane of a responsive cell. An inactive decoy receptor also exists in a membrane and soluble form, IL-1RII, and is considered anti-inflammatory by acting as a sink for IL-1β. In the introduction to inflammation above, the several pathways that are activated when by the IL-1R were discussed. The broad spectrum of IL-1R activation is mirrored in the literature where this cytokine has been implicated in various functions and pathological conditions. The stimulation of neurons in the hypothalamus by IL-1β induces some of the physiological and behavioural changes associated with inflammation, the sickness syndrome, which is associated with decreased energy and appetite as well as increased body temperature and hyperalgesia [124]. The importance of IL-1β for the regulation of neuronal plasticity has been mentioned. IL-1β also has functions as a growth factor in the development of the nervous system [125].

3.3.3 **IFNγ**

Interferon-γ is the second archetypical pro-inflammatory cytokine. It is mainly produced by activated T_{H1}-cells and is considered the prime activator of cells of the monocyte lineage, stimulating them to attain a pro-inflammatory T_{H1} phenotype. There are two types of receptors for IFNγ (IFNGR1 and IFNGR2) which upon binding of the ligand first homodimerize, after which two IFNGR1 and IFNGR2 homodimers associate, thus forming a heterodimer formed by association of two different homodimers. The assembly of this complex makes signal transduction through Jak1/2 – STAT1 possible, resulting in the transcription of a number of genes, among them one of the subunits of the κB complex, which is expressed in low levels in basal conditions. The effects of IFNγ on the activation of iNOS and expression of MHC)I and II has been discussed in the introduction of this thesis.

3.3.4 DHA and EPA

Docosahexanoic acid (DHA) is an omega-3 fatty acid that has received lot of attention in recent times for its proposed beneficial effects on several conditions related to inflammation, in the periphery as well as in the CNS. Of special interest are the metabolites that result from biotransformation of DHA. Neuroprotectin D1 is a fatty acid derived from DHA that has been shown to protect neurons and have anti-inflammatory properties [67; 126]. Another derivative of DHA is maresin, which also has been shown to be anti-inflammatory and to stimulate phagocytosis [127]. Neuroprotectin D1 and maresin are believed to mediate the resolution stage of inflammation. Eicosapentaenoic acid (EPA) is also an omega-3 fatty acid with anti-inflammatory properties [126]. Resolvins are derivatives of EPA and believed to mediate the effects of EPA [67]. As the name implies, resolvins favour the resolution of inflammation and as such can be neuroprotective by limiting inflammation in the CNS. It should also be noted that there are pathways between the metabolism of DHA and EPA, permitting the conversion of metabolites from each pathway to metabolites of the other.

Effects of DHA and EPA can also be mediated by influence on membrane dynamics and fluidity. DHA is abundant in the brain gray matter, while EPA is not. However, the conversion potential between the two fatty acids, and the results from studies showing that EPA has beneficial effects on disorders in the CNS, suggest an important role of EPA in the CNS.

Both EPA and DHA can activate the PPAR family of proteins, which can contribute to their anti-inflammatory effects [128].

3.3.5 EPAX and placebo

EPAX 1050TG™ is a product of Pronova Biocare A/S Lysaker, Norway. It is a capsule which contains 430 mg DHA and 150 mg EPA. The placebo capsules contained 1 g corn oil which includes 0.6 mg of linoleic acid.

3.3.6 α-Melanocyte stimulating hormone (α-MSH)

α-MSH is a thirteen amino acid neuropeptide that has been shown to have potent anti-inflammatory properties. Neuroprotection by α-MSH has been reported in a number of studies on animals, but data from experiments on human tissues are scarce [68; 129]. The effects of α-MSH on human tissues have not been studied extensively. α-MSH binds to melanocortin receptors of which there are five subtypes (MCR1R, MCR2R, etc) [130]. All subtypes have been found in the brain, except MCR2R, which is a receptor for adrenocorticotrophic hormone

(ACTH), exclusively. MCR2R is expressed in the adrenal cortex where its activation stimulates the secretion of cortisol. Signal transduction through melanocortin receptors is mediated by stimulatory G-proteins, which activates adenylyl cyclase (AC), followed by production of cAMP. cAMP activates protein kinase A (PKA) which in turn activates the cAMP-response element binding (CREB)-protein. The activated CREB-protein then activates genes with anti-apoptotic functions, and functions positively regulating neuronal plasticity. The anti-inflammatory effect of α-MSH is proposed to be mediated by the inhibition of NF-κB and the induction of anti-inflammatory cytokines [131; 132; 133].

4 RESULTS AND DISCUSSION

In **paper I** the human CHME3 microglia cell line was characterized with regard to inflammatory responses after incubation with $A\beta_{1\text{-}40}$. The effects of IFNγ and α-MSH by themselves, and together with $A\beta_{1\text{-}40}$, were also studied. The presence of MCR:s was also investigated. The inflammatory response was measured by the secretion of IL-6 and cell proliferation.

In **paper II** we widened the scope to include inflammatory responses that can be beneficial. As in paper I, the inflammatory response was measured by the secretion of IL-6. The phagocytosis of $A\beta_{1\text{-}42}$ and the secretion of BDNF were introduced as positive outcomes of microglial activation. ICC was also performed to elucidate the phenotype of microglia that phagocytose $A\beta_{1\text{-}42}$.

Paper III is part of the OmegAD-study which is an intervention study aimed at investigating the potential of omega-3 fatty acids for the treatment of AD. The participants in the OmegAD-study were patients diagnosed as suffering from mild to moderate AD based on DSM-IV classification. Plasma and CSF were analysed for the levels of markers related to inflammation and AD at base line, and after 6 months of treatment with the omega-3 preparation or placebo.

To further study the effects of omega-3 fatty acids on inflammation in the CNS we performed an *in vitro* study that resulted in **paper IV**. The methodology in paper II was used to study the effects of the omega-3 fatty acid DHA on phagocytosis of $A\beta_{1\text{-}42}$, secretion of IL-6 and BDNF, and the inflammatory phenotype of the CHME3 microglia. The effects of DHA on phagocytosis of inert latex beads and other parameters were also studied. In the studies on latex beads, the secretion of a set of T_{H1} and T_{H2} cytokines was also studied.

In the **Preliminary results** we describe the results obtained when investigating the neuroprotective and plasticity-promoting effects of α-MSH on differentiated human neuronal cells. Cell proliferation and cell death, as well as the degree of apoptosis were measured. The effects on plasticity were analysed with ICC and Western blotting.

4.1 CHARACTERIZATION OF HUMAN CHME3 MICROGLIA WITH REGARD TO INTERACTIONS WITH $A\beta_{1-42}$ AND INFLAMMATION

In **paper I** and **II,** the human CHME3 microglia were analysed with regard to their reactions to incubation with $A\beta_{1-42}$ and a set of immunomodulatory substances. The responses in terms of secretion of the pro-inflammatory cytokine IL-6 and the neurotrophin BDNF, cell proliferation and cell death, as well as phagocytosis of $A\beta_{1-42}$ will be discussed.

4.1.1 Secretion of IL-6 from human CHME3 microglial cells

As a cytokine that appears in the later part of the acute phase and that is the effector of inflammation on several tissues, IL-6 can be used as an indicator of inflammatory responses.

4.1.1.1 Secretion of IL-6 upon incubation with pro-inflammatory cytokines (Paper I, II, IV)

Pro-inflammatory stimulation in the form of IL-1β and IFNγ increased IL-6 secretion, as expected. The most vigorous secretion was induced by IL-1β (50 ng/ml) which after 48 h of incubation increased the levels of IL-6 in the medium around 20-30 times over the levels observed under basal conditions.

4.1.1.2 Secretion of IL-6 upon incubation with $A\beta_{1-40}$ or $A\beta_{1-42}$ (Paper I, II, IV)

$A\beta_{1-40}$ at 5 μM induced a significant increase in IL-6 secretion of 40% over vehicle (**paper I**). There was no significant effect on IL-6 secretion from the cells upon incubation at 2 μM (5 μg/ml fluorophore-labelled $A\beta_{1-42}$ (**paper II**). Although the difference in concentration of the Aβ in the two papers, we believe that the major cause is a difference in the activating properties of $A\beta_{1-40}$ and $A\beta_{1-42}$, which has been suggested in previous studies [134]. The preparation procedure and the solvent used are also factors that may influence the outcome of experiments on Aβ. A key event appears to be the oligomerisation of Aβ which creates neurotoxic forms of Aβ, while fibrillisation creates forms that are less toxic [135]. Aside from neurotoxicity, the immune-activating properties of Aβ have also been shown to be dependent on the aggregational form of Aβ in studies in rat microglia [134]. An explanation to the differences in effects may lie in the appearance of structures resulting from differential aggregation of Aβ dissolved or prepared in different conditions. The appearance of structures that act as Ca^{2+}-ionophores, or ligands to receptors that mediate activation is possible.

IL-6 has been shown to be increased in the brains of AD patients, rendering IL-6 a prime suspect of being involved in the pathology of this disease [136]. There are, however, beneficial effects of IL-6 on neurons. Studies *in vitro* have shown that IL-6 can promote neurite outgrowth and neuronal survival [137; 138; 139; 140]. However, in a study by Qiu et al it was shown that rat cortical neurons were more severely affected by incubation with N-methyl-D-aspartate (NMDA) and $A\beta_{1-42}$ when IL-6 was present [141]. In addition, IL-6 has been shown to inhibit neurogenesis, and promote astrocyte differentiation from neuronal precursor cells [104; 142; 143]. Furthermore, as one of the "final" cytokines in the pro-inflammatory cytokine cascade, IL-6 is an effector of many of the harmful responses of inflammation [144]. It is most probable that inhibition of the increased secretion of IL-6 observed in the AD brain would be beneficial. To conclude, CHME3 microglia respond to IL-1β and IFNγ stimulation with increased IL-6 secretion, thus showing a normal macrophage-like response to this type of stimulation. The cells respond to $A\beta_{1-40}$ with an increase in their secretion of IL-6 in a dose-dependent manner, while $A\beta_{1-42}$ was without effect at the concentrations tested.

4.1.2 Secretion of BDNF from human CHME3 microglial cells

The secretion of BDNF was analysed in **paper II** and **IV**. An increased secretion of BDNF is a positive outcome of inflammation that has been reported in several studies. The focus has been on the secretion of BDNF from neuronal cells, while microglial secretion has been receiving relatively little attention. Neuronal secretion of BDNF is most likely more important than glial secretion for the functioning of neurons due to the temporal and spatial qualities required for consolidation of memory. However, one should not discard the possibility that glial secretion can play a beneficial role in neurodegenerative conditions by increasing the interstitial concentration of BDNF.

4.1.2.1 Secretion of BDNF upon incubation with pro-inflammatory cytokines (Paper I, II, IV)

A presumably detrimental reduction in the secretion of BDNF was seen when the microglia were incubated with IFNγ (50 ng/ml) (**paper II**). As discussed above, this is in discord with other reports showing an increase in growth factor secretion in inflammation. However, in the experiments presented in this thesis the microglia were stimulated with quite high concentrations of IL-1β and/or IFNγ, which are only two of the components of the inflammatory cascade, and it may be that the situation in the brain is different. Nevertheless, it

suggests a pathological effect of IFNγ that is relevant for AD and that may play a role in the pathology and pathogenesis of this disease. The effects of IL-1β on the secretion of BDNF were small and co-incubation with IFNγ produced results similar to those obtained with IFNγ alone. In **paper IV** a small, yet significant stimulatory effect on BDNF secretion could be observed upon incubation with IL-1β and latex beads. In view of the lack of effect upon stimulation with $A\beta_{1-42}$ and IL-1β, and when stimulating the cells with IL-1β alone, it can be speculated that phagocytosis of the latex beads modulated the response to IL-1β into increased secretion of BDNF.

4.1.2.2 Secretion of BDNF upon incubation with $A\beta_{1-42}$ (paper II, IV)

In **paper II,** a negative effect on the secretion of BDNF was observed upon incubation with $A\beta_{1-42}$. At the concentration of 1 μg/ml the results were inconsistent, *i.e.* in the Protollin series of experiments a significantly lower level of BDNF was observed, while in the cytokine series of experiments the effect was not statistically significant. In Fig. 3 the data from all experiments with 1 μg/ml $A\beta_{1-42}$ are combined, showing a significant reduction in the secretion of BDNF from CHME3 microglia. At the concentration of 5 μg/ml, $A\beta_{1-42}$ significantly decreased microglial secretion of BDNF to 50% that of control. No additive effect was observed when the microglia were incubated with $A\beta_{1-42}$ and IFNγ. What is the mechanism by which $A\beta_{1-42}$ lowers the secretion of BDNF from microglia? Tong et al found that $A\beta_{1-42}$, in approximately the same sublethal range of concentrations used in this thesis, impaired the phosphorylation of CREB, thereby reducing activation of genes with a CRE motif [145; 146]. Furthermore, they found that this effect was separate from the acute neurotoxicity, and related to the impairment of neuronal plasticity. However, impairment of neuronal plasticity, can certainly contribute to the neurodegeneration in the AD brain by depriving neurons of trophic stimulation. It is the author's opinion that the extremely high concentrations used to kill neurons *in vitro* are irrelevant for AD, since neurons would never or rarely encounter Aβ at those concentrations *in vivo*. The notion that incubation with a high concentration of Aβ for a short time can equal exposure to low concentrations for years is far-fetched and unrealistic in the author's opinion. In fact, the "secondary" effects of Aβ, such as inflammation and negative regulation of plasticity and growth factors, may be pivotal in the pathogenesis of AD.

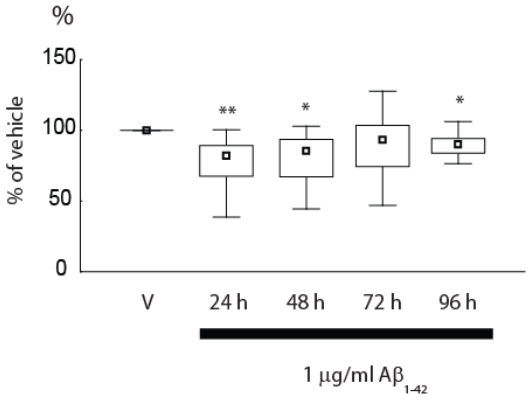

Fig. 3. Combined data from the cytokine and Protollin experiments showing a reduction of microglial BDNF secretion upon incubation with 1 μg/ml $A\beta_{1-42}$. The data are expressed as % of control vehicle set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by * ($p < 0.001$) and ** ($p < 0.0000001$).

4.1.3 Cell proliferation and cell death

A simple assay for activation of hematopoietic cells is to assess their proliferation which in most cases is increased upon activation. It is unclear if this is applicable for primary cultures of microglia, which represent a very differentiated cell and as such have a limited proliferation capacity. The CHME3 cell line, on the other hand, is very proliferative as a consequence of transformation.

4.1.3.1 Microglial proliferation and cell death upon incubation with pro-inflammatory cytokines (paper I, II)

Long-term (96 h) incubation with IFNγ decreased cell proliferation drastically and microscopical inspection of the cultures at this stage showed cells in severe stress. IFNγ is known to increase oxidative stress by the activation of iNOS and lysosomal oxidases which may explain this finding. Notably, IFNγ produced no detectable detrimental effects on microglial viability at the earlier time points, suggesting an accumulation of toxicity that fits well with a hypothetical increase in oxidative stress.

4.1.3.2 Microglial proliferation and cell death upon incubation with $A\beta_{1-40}$ or $A\beta_{1-42}$ (paper I, II)

$A\beta_{1-40}$ was found to decrease microglial viability at the lowest concentration tested (5 μM) when incubated for 24 h. There were no effects of $A\beta_{1-42}$ on cell viability or cell death at the concentrations tested.

4.1.4 Phagocytosis

Removal of debris and pathogens from the tissue to restore homeostasis is an important activity of cells of the monocyte lineage. To analyse the phagocytic capacity of CHME3 microglia the cells were incubated with fluorophore-conjugated $A\beta_{1-42}$ or latex beads and analysed with flow-cytometry. ICC using antibodies against lamp-2 were performed to establish phagocytosis of $A\beta_{1-42}$ by showing the lysosomal location of intracellular $A\beta_{1-42}$ using confocal microscopy, thus indicating that the phagocytosed $A\beta_{1-42}$ was destined for degradation (see paper II).

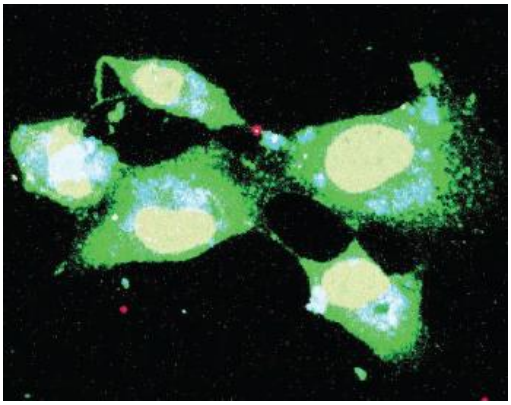

Fig. 4. This microphotograph, taken in a confocal microscope, shows fixed human CHME3 microglia that have been incubated with biotinylated $A\beta_{1-42}$. After fixation the cells were stained with antibodies against IL-1β. $A\beta_{1-42}$ was visualized by incubation with streptavidin-AMCA (blue).

4.1.4.1 Phagocytosis of latex beads (paper IV)

Latex beads are considered to be relatively inert from the perspective of activation. At 48 h of incubation, an average of 13.1% (+/- 5.5% SD) of the cells displayed phagocytosis of latex beads, representing the basal level.

4.1.4.2 Phagocytosis of $A\beta_{1-42}$ (paper II, IV)

The first visible sign, as analysed in inverted fluorescence microscope, of cellular uptake of $A\beta_{1-42}$ was observed at about 4 h after incubation. The phagocytosed $A\beta_{1-42}$ first appeared as small, vague clouds of fluorescence. Later, small and round dots of strong fluorescence started to appear. When inspecting the cells in microscope, it was clear that only a subset of CHME3 microglia were actively phagocytic with regard to $A\beta_{1-42}$. This was confirmed when the cells were analyzed with flow-cytometry revealing that an average of 3.6% (+/- 1.1% SD) of the CHME3 microglia contained the fluorophore-conjugated $A\beta_{1-42}$ in the DHA series of experiments (**paper II**). Hence, the number of cells showing uptake of latex beads is higher than the number of cells showing uptake of $A\beta_{1-42}$ in comparable conditions, indicating a

difference in the mechanism of phagocytosis of the two objects. Fig. 4 shows IL-1β-positive CHME3 microglia with uptake of $A\beta_{1-42}$.

4.1.4.3 Effect of pro-inflammatory cytokines on phagocytosis (paper II, IV)

Pre-incubation with IFNγ significantly stimulated the phagocytosis of $A\beta_{1-42}$. This may be mediated STAT proteins, since activation of these transcription factors has been associated with increased phagocytic activity [147]. However, since the effect became apparent at a late time point, it is reasonable to suggest that mediators secondary to IFNγ may be important. A stimulatory effect on phagocytosis by IFNγ is also in accord with other cellular effects associated with activation with IFNγ: upregulation of antigen-presentation, increase in lysosomal activity and activation of iNOS [147; 148; 149; 150; 151; 152]. IL-1β did not increase the phagocytosis of $A\beta_{1-42}$ or latex beads, and the combination of IFNγ and IL-1β produced effects similar to those seen upon incubation with IFNγ alone.

4.1.5 Microglial phenotype and relation to phagocytosis

The phenotype of CHME3 microglia was analysed in **paper II** and **IV**. In **paper II**, Protollin was found to increase the number of IL-1β positive phagocytic cells. In **paper II**, we interpreted a cellular signal of a secreted inflammatory protein with the assumption that an increase over basal levels is a sign of activation. Thus we concluded that Protollin activated the cells, which is in line with Protollin being a TLR2 agonist. However, in **paper IV**, where both the secretion of IL-6 and cellular IL-6 were analyzed, it was found that cultures with a high secretion of IL-6 displayed a low number of cells with a cellular expression of IL-6. It may be that cells with a high secretory activity are being emptied of the secretory product, thus showing a low signal. More experiments are needed to elucidate the relationship between the cellular and secreted levels of secretory proteins but for now, no conclusion can be drawn about the activation status of the CHME3 microglia based on the cellular presence of IL-1β.

4.1.5.1 Differential phenotype of phagocytic and non-phagocytic microglia (paper II and IV)

Among cells showing phagocytosis of $A\beta_{1-42}$ the proportion of iNOS-positive cells was larger than among non-phagocytic cells. Furthermore, a similar pattern was observed with regard to the expression of IL-1RI. This leads us to believe that microglia with an inflammatory phenotype are more prone to phagocytose $A\beta_{1-42}$. Interestingly, there were differences in the phenotype of microglia showing phagocytosis of $A\beta_{1-42}$ *vs* microglia that phagocytose latex

beads. Among cells showing phagocytosis of $A\beta_{1-42}$, there was a significantly higher proportion of cells being positive for iNOS and IL-1RI. In the case of latex beads, the pattern was completely reversed. Latex beads are relatively inert from an activation perspective and are many times larger than the aggregates/deposits of $A\beta_{1-42}$ observed in the cultures as judged by microscopical inspection. Hence, it is not unreasonable to assume that the molecular mechanisms of recognition and phagocytosis are different, depending on the objects.

4.2 IMMUNOMODULATION OF RESPONSES RELATED TO NEUROPROTECTION AND NEURODEGENERATION

In our human cellular models of phagocytosis and neurodegeneration we tested a set of substances for their ability to stimulate beneficial activities and to inhibit harmful responses. The effects of an intervention with omega-3 fatty acids on inflammatory factors in AD-patients were also studied.

4.2.1 Modulation of phagocytic activity by Protollin and DHA

In **paper II**, the ability of Protollin to stimulate phagocytosis of $A\beta_{1-42}$ by CHME3 microglia was tested. The microglia were pre-stimulated with 0.001 – 1 μg/ml protollin for 24 h after which fluorophore-labelled $A\beta_{1-42}$ was added to the cultures and phagocytosis of $A\beta_{1-42}$ was analysed at 24 – 96 h after addition of $A\beta_{1-42}$. In **paper IV**, the effects of the omega-3 fatty acid DHA on phagocytosis of $A\beta_{1-42}$ and latex beads were investigated. The cells were incubated with 0.01 – 2.5 μM DHA together with fluorophore-conjugated $A\beta_{1-42}$. Alternatively, 0.05 μM DHA, alone or together with IL-1β, was added to the microglial cultures together with latex beads.

In preliminary studies on mouse microglia it was found that 24 h of pre-incubation with Protollin was most effective for stimulation of phagocytosis of $A\beta_{1-42}$. The interpretation of this result was that the effects on phagocytosis by Protollin were mediated by the secretion of secondary products, which affect phagocytosis by auto- and paracrine stimulation. The effect of Protollin on phagocytosis of $A\beta_{1-42}$ by the human CHME3 microglia was limited. A small but significant increase was obsered in the late phase of the experiment upon treatment with the lowest concentration of protollin (0.001 μg/ml). Several studies have found that TLR2 ligands can stimulate phagocytosis [153; 154]. However, in these studies, the phagocytosis of bacteria or tissue debris was the focus and there may be different pathways or mechanisms involved in the phagocytosis of Aβ. Recently, it has been suggested that TLR2 activation is, in fact, vital for

activating microglia into performing the functions of inflammation, such as phagocytosis [155]. It has also been found that TLR2 is vital for microglia activation by Aβ [156]. In the present studies, the number of phagocytic microglia was quite low in basal conditions compared to other studies on the phagocytosis of $A\beta_{1-42}$ [157; 158]. However, those studies were performed on non-human microglia and the number of phagocytic cells was in some cases quantified with other methods, which can explain the difference. However, in a study on aged human primary microglia, Familian et al found a comparable level of phagocytosis, as analysed with flow-cytometry [159],

DHA was found to have a stimulatory effect on phagocytosis of $A\beta_{1-42}$ at 6 h. This effect seemed to linger at 24 h, although the result was not significant. The short time of action of DHA may be due to oxidation of DHA or its downstream products. In addition, the poor solubility of DHA or its downstream products in cell-culture medium, may make them stick to the culture dish, thus reducing their effective time of action. The phagocytosis of latex beads was only analyzed at 48 h. At this time point, there was no effect on phagocytosis by the treatment with DHA, IL-1β or the combination of the two. In agreement with this result, there was no effect of DHA at any concentration on the phagocytosis of $A\beta_{1-42}$ at this time point.

4.2.2 Effects on microglial activation by α-MSH

The effects of α-MSH on CHME3 microglia activated by $A\beta_{1-40}$ or IFNγ were analysed with regard to the secretion of IL-6 and cell proliferation. There was no anti-inflammatory effect of α-MSH with regard to the secretion of IL-6. On the contrary, we found a dose-dependent stimulatory effect on IL-6 secretion by α-MSH by itself. The absence of inhibition on IL-6 secretion may be explained by the absence of a κB binding site on the IL-6 gene, and the stimulation may be explained by the presence of a CRE motif. CRE is activated by the CREB protein, which in turn is activated by PKA, which is the primary signaling pathway that is activated by α-MSH. PKA is activated by cAMP, which has been implicated in the inhibition of NF-κB, thus mediating the anti-inflammatory effect of α-MSH [160; 161]. In accord with this result, Jun et al found that activation of type 2 and 5 MCR:s stimulated the secretion of IL-6. We demonstrate that human CHME3 microglia express the MCR type 1, 3, 4 and 5. Melanocortin receptor has also been shown to activate PKC and the PI3K pathway in addition to activation of AC [162; 163; 164; 165]. The involvement of PKC in the regulation of inflammation is well-known and NF-κB activation was shown to occur downstream of PI3K

[166]. It seems clear therefore that α-MSH may act in a pro-inflammatory manner under the some circumstances.

4.2.3 Effects on microglial phenotype by Protollin and DHA

To determine the effect on the phenotype of human CHME3 microglia upon treatment with Protollin or DHA, the cellular immunoreactivity to a set of markers of the whole microglial population was analysed, as well as the differential immunoreactivity of phagocytic and non-phagocytic cells.

4.2.3.1 IL-1β (paper II, IV)

Pretreatment with Protollin was found to increase the number of IL-1β-positive phagocytic cells compared to cultures treated with $A\beta_{1-42}$ alone at the 24 and 48 h time points. Cultures treated with $A\beta_{1-42}$ alone, the phagocytic microglial population displayed a higher expression of IL-1β compared to non-phagocytic cells. This difference was seen at 6 h but disappeared at 24 h, in accord with the results in **paper II**. In contrast, at this time point cultures treated with DHA together with $A\beta_{1-42}$ displayed a significantly larger number of IL-1β-positive microglia that were phagocytic of $A\beta_{1-42}$ (see Fig. 4).Treatment with DHA alone increased the total number of IL-1β-positive cells at 24 h. Regarding the effect of DHA on the differential immunoreactivity, of phagocytic and non-phagocytic cells, it was found that at 48 h there were a significantly higher number of phagocytic microglia expressing IL-1β in all the treatments. There was no effect on the total number of IL-1β-positive cells in the experiments on latex beads upon any of the treatments, nor were there any differences between phagocytic and non-phagocytic cells. Interestingly, the cultures treated with 50 ng/ml IL-1β for 48 h did not display any difference in the number of cells showing immunoreactivtiy to IL-1β compared to the other treatments.

4.2.3.2 iNOS (paper II, IV)

A rather consistent result upon incubation of the microglia with $A\beta_{1-42}$ was the significantly higher number of iNOS-positive cells in the phagocytic population compared with the non-phagocytic. In **paper II**, this pattern of differential expression of iNOS was seen during the whole experiment, including the latest time point (96 h). The pattern of differential expression disappears with time upon incubation with Protollin, with equal distribution of iNOS between the phagocytic and non-phagocytic microglia at the latest time point at 96 h. In **paper IV**, there were more iNOS-positive cells in the phagocytic population in cultures treated with Aβ1-42

alone at 48 h, in accord with the results seen in **paper II**. Similarly, treatment with DHA abolished the difference in expression between phagocytic and non-phagocytic microglia. Interestingly, in experiments with latex beads, the expression of iNOS was more abundant in the non-phagocytic population, in contrast to the result upon incubation with $A\beta_{1-42}$.

4.2.3.3 IL-1RI (paper II, IV)

With regard to phagocytosis of $A\beta_{1-42}$, there were significantly more IL-1RI-positive cells in the phagocytic population in all treatments and time points. At the latest time point at 96 h, the difference between the populations appeared to abate.

4.2.3.4 TNFα (paper IV)

The cellular expression of TNFα was analysed in the experiments on latex beads. The total immunoreactivity of the microglia was not altered upon any of the treatments. However, there was higher expression of TNFα in non-phagocytic cells than in phagocytic cells upon treatment with 0.05 μM DHA for 48 h.

4.2.3.5 IL-4R (paper IV)

The cellular expression of IL-4R in the experiments on latex beads was not altered upon any of the treatments, nor did any of the treatments affect the differential immunoreactivity to IL-4R of the phagocytic and non-phagocytic cells.

4.2.3.6 IL-6 (paper IV)

Analysis of the cellular expression of IL-6 in the experiments on latex beads showed a significantly higher expression in phagocytic cells in the cultures treated with DHA and IL-1β for 48 h. Microglia treated with IL-1β alone were also suggestive of this imbalance, but the difference was void of statistical significance.

4.2.4 Preliminary results

Studies on our model of neuronal cell death in differentiated human neuronal SY5Y cells showed a protective effect of α-MSH on necrosis induced by ionomycin. Furthermore, we have preliminary data that indicate a positive effect of α-MSH on apoptosis induced by staurosporine.

4.2.4.1 Effects of α-MSH on cell death induced by ionomycin

To evaluate the protective properties of α-MSH on necrotic cell death differentiated SY5Y cells were incubated with 1 μM ionomycin, alone or together with 0.01 – 1 μM α-MSH for 48 h. Microscopical inspection of the cultures at this timepoint showed that 1 μM ionomycin caused extensive cellular stress indicated by retracted neurites, rounded-up cells, and cellular deattachment. In all cultures where treated with α-MSH, in all concentrations examined, there was visibly less extensive cellular stress. These observations were mirrored in the results from the LDH-assay which confirmed extensive necrotic cell death by 1 μM ionomycin (40.5% of positive control for ionomycin compared to 25% of positive control for vehicle, $p < 0.05$) (Fig. 4). The LDH-assay also confirmed protection by α-MSH in the concentration 0.1 μM with a LDH-activity comparable to vehicle (23% of positive control, $p < 0.05$ when comparing with ionomycin alone).

4.2.4.2 Effects of α-MSH on apoptosis and cell death induced by staurosporine

Protective effects of α-MSH on cellular stress induced by staurosporine were investigated by incubating differentiated SY5Y cells with 250 and 500 nM staurosporine, alone or together with 0.001 – 1 μM α-MSH for 48 h. For analysis of apoptosis with the TUNEL-assay, differentiated SH SY5Y cells were incubated for 48 h with 500 nM staurosporine, alone or together with 0. 1 or 1 μM α-MSH.

4.2.4.2.1 Cell viability

Preliminary data from the MTT-assay indicate a substantial negative effect of 250 and 500 nM staurosporine on differentiated SY5Y cells. Cell viability was decreased to 10% of vehicle control by 250 and 500 nM staurosporine. α-MSH increased cell viability to 38% when the cells were incubated with a concentration of 1 μM together with 250 nM staurosporine and to 31% when 1 μM α-MSH and 500 nM staurosporine were added to the cells.

4.2.4.2.2 Cell death

Preliminary data from the LDH-assay indicate necrosis (or late apoptosis) in the cultures treated with 250 and 500 nM staurosporine. Both concentrations increased LDH activity in the medium to around 40% of positive control compared to 24% of positive control in the vehicle group. The preliminary results indicate a slightly protective effect of α-MSH in the highest

concentration: when 1 mM α-MSH was incubated with 500 nM staurosporine, the LDH-activity decreased from 44 to 34%.

4.2.4.2.3 Apoptosis

The number of apoptotic cells in the cultures was determined with the TUNEL-assay. A cell suspension from each treatment was subjected to the TUNEL-method. The number of TUNEL-positive cells was then quantified with flow-cytometry. Preliminary data indicate a protective effect of α-MSH to staurosporine-induced apoptosis. In the culture treated with 500 nM staurosporine alone, 69% of the cells were identified as TUNEL-positive compared with 46% in the vehicle group. Treatment with 500 nM staurosporine together with 0.1 μM α-MSH reduced the number of TUNEL-positive cells to 61% and 1 μM α-MSH reduced the percentage even further to 51%.

4.2.4.3 Effects of α-MSH on reduction in cell viability induced by $A\beta_{25-35}$

To investigate protective effects of α-MSH on Aβ-mediated toxicity differentiated SY5Y cells were incubated with 5, 25 and 50 μM $A\beta_{25-35}$ alone or together with 1 μM α-MSH for 24 h.

4.2.4.3.1 Cell viability

Preliminary data from the MTT-assay suggest a decrease in cell viability by 5 μM $A\beta_{25-35}$ to 71% of vehicle and a slight protective effect by 1 μM α-MSH. Incubation of the cells with 5 μM $A\beta_{25-35}$ and 1 μM α-MSH increased cell viability to 83% of vehicle. Similarly, incubation with 25 μM $A\beta_{25-35}$ and 1 μM α-MSH resulted in increased cell viability to 82% from the 69% of vehicle that was induced by incubation with 25 μM $A\beta_{25-35}$ alone. Incubation with 50 μM $A\beta_{25-35}$ alone decreased cell viability to 64% of vehicle and this was increased to 69% by 1 μM α-MSH.

The protective effect of a substance on apoptosis and necrosis, respectively, may be attributed to different mechanisms. α-MSH can protect cells from apoptosis by the increased production of cAMP which is mediated by all MCR:s. cAMP activates PKA and ERK [131; 163], which are known to be protective when activated. Downstream of PKA is the activation of CRE-containing genes, among which are several anti-apoptotic members of the bcl-2 family and BDNF. The mechanism for protection against necrosis is more unclear. Necrosis is not a controlled process but rather the breakdown of vital processes due to extreme stress in the form

of altered pH, osmotic pressure, physical damage and ionic imbalance. Of course, apoptosis can lead to necrosis if the stress is too severe, and it is not unreasonable to assume that the two processes interact. For example, an anti-apoptotic substance may to some degree rescue cells that are exposed to a type of stress dominated by necrosis by relieving the cells from the additional stress caused by the apoptotic process that most probably is working in parallel, consuming valuable ATP in the process. It can also be speculated that the activation of ERK, or other kinases implicated in the activity of cells, increases survival by increasing the activity of signalling pathways that support the integrity of a cell (actin remodelling, membrane maintenance, etc).

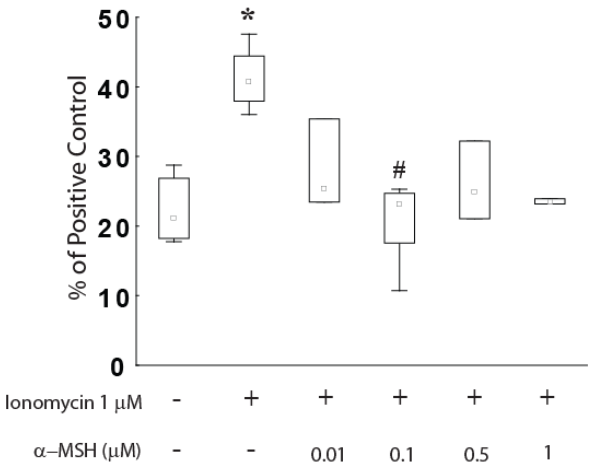

Fig. 4. Protection by α-MSH from neuronal cell death induced by ionomycin. Differentiated SY5Y cells were incubated with 1 μM ionomycin, alone or together with 0.01- 1 μM α-MSH and analysed with LDH-assay. The data are expressed as % of positive control (vehicle-treated cells lysed with 2% Triton-X100) and shown as median ± percentiles (25% - 75% and 10% - 90%). * indicates statistically significant difference from vehicle ($p < 0.05$). # indicates statistically significant difference from 1 μM ionomycin ($p < 0.05$).

4.3 EFFECTS ON MARKERS RELATED TO INFLAMMATION AND AD IN AD-PATIENTS AFTER SUPPLEMENTATION WITH OMEGA-3 OR PLACEBO

In **paper III** the effects of the intervention on AD-patients with omega-3 fatty acids on the levels of markers related to inflammation and AD in CSF and plasma were analysed. There was no significant effect on the levels of any of the markers analysed after 6 months of treatment with EPAX or placebo. At baseline, a positive correlation was found between the levels of the soluble IL-1 receptor (sIL-1RII) and $A\beta_{1-42}$. However, after 6 months of treatment with EPAX or placebo, this correlation was lost. sIL-1RII is a secreted receptor which is lacking the intracellular signalling domain of the functional IL-1RI and is therefore called a decoy receptor [167]. sIL-1RII can therefore be considered an anti-inflammatory, or even a pro-resolving factor. Secretion of sIL-1RII from monocytic cells is induced by anti-inflammatory cytokines such as IL-4 and IL-13 which also induce the secretion of the endogenous IL-1R antagonist (IL-1Ra) [168]. sIL-1RII can thus be viewed as part of the T_{H2} polarisation mechanism by sequestering IL-1β and blocking IL-1α. Since human blood contains high concentrations of sIL-1RII under basal conditions it can also exert a dampening effect when the immune system is under pathogenic challenge, thus preventing sepsis or other extreme and dangerous conditions. The interpretation of this result discussed in **paper III** is that an increase in the levels of $A\beta_{1-42}$ in the CSF drives an inflammatory response in the brain and sIL-1RII is secreted in higher levels as a protective response. Indeed, the levels of sIL-1RII were higher in a population of patients with mild to moderate AD [169]. However, an increase in the levels of $A\beta_{1-42}$ in CSF has been linked to an improvement in the condition. An impaired transport of $A\beta_{1-42}$ into the CSF has in fact been proposed as a pathogenic mechanism in AD. An alternative explanation, that appears more plausible in the light of data from recent studies, is that high levels of $A\beta_{1-42}$ in the CSF is related to a low degree of inflammation in the CNS, which is expressed in the result from **paper III** as an increase in the anti-inflammatory sIL-1RII due to lack of T_{H1} suppression by T_{H2} mediators. The lack of effect on the markers analysed in this study can be explained by the timing of intervention. Several of the studies on intervention on established AD has been yielded negative results. It may be that it is too late for intervention with omega-3 when the disease has progressed to a symptomatic stage. At this stage it is believed that the loss of neurons has been going on for some time and treatment with omega-3 may be ineffective.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The primary aim of this thesis was to investigate the possibility of modulating inflammation in a way that increases beneficial activities while downregulating the harmful ones for the treatment of neurodegenerative conditions, particularly with regard to AD. For this purpose a human microglial cell line, CHME3, was characterized for the use as a model of AD-related inflammation in the CNS. This resulted in the establishment of a model of phagocytosis of $A\beta_{1-42}$ by human microglia. In this model a number of immunomodulatory compounds were tested for their capacity of stimulating phagocytosis of $A\beta_{1-42}$. Furthermore, other responses related to neurodegeneration and inflammation were studied upon different treatments.

To summarise all the studies presented in this thesis, the author would like to present what he believes are the major findings:

- $A\beta_{1-42}$ may play a new pathological role by reducing the secretion of BDNF from human microglia
- Only a subset of human CHME3 microglia are phagocytic of $A\beta_{1-42}$
- Microglia that phagocytose either $A\beta_{1-42}$ or latex beads are of different phenotype, or alternatively, attain a different phenotype upon phagocytosis
- Human CHME3 microglia exhibited several differences in responses compared to animal models of microglia
- Inflammation related to AD in the form of IFNγ induces an activation of microglia that is associated with increased phagocytosis, increased secretion of IL-6, impaired neuroprotective activities in the form of reduced secretion of BDNF, and microglial cell death
- Omega-3 fatty acid supplementation in the form of EPAX may be ineffective for the treatment of established AD treated with acetylcholine esterase inhibitors, based on the lack of detectable changes in the levels of $A\beta_{1-42}$, t-tau and p-tau in the CSF, and in the levels of inflammatory markers in CSF and plasma
- The omega-3 fatty acid DHA may be useful for the prevention, or treatment, of AD by stimulating microglial phagocytosis of $A\beta_{1-42}$ and decreasing the levels of inflammatory cytokines

- Treatment with α-MSH may be beneficial for the outcome of neurodegenerative conditions in humans, although the unexpected pro-inflammatory effect on human microglia should be viewed with caution.

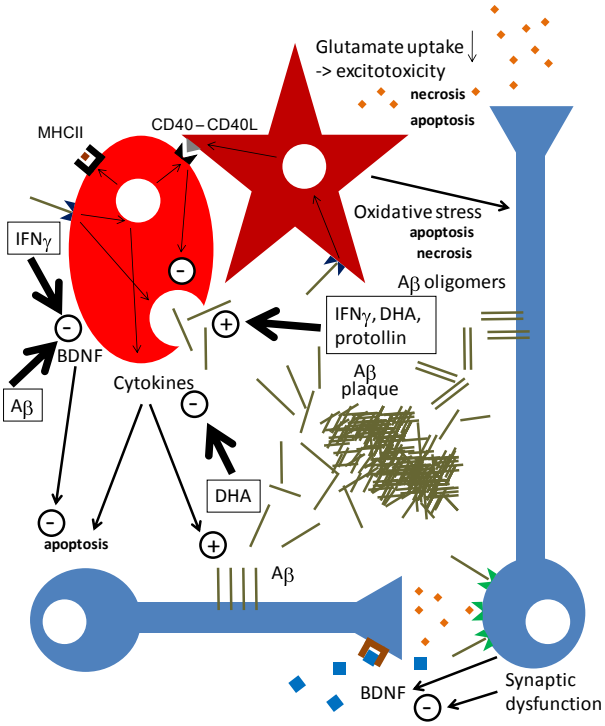

Fig. 5. An updated version of Fig. 1 based on the results presented in this thesis. $A\beta_{1\text{-}42}$ decreases microglial secretion of BDNF in concentrations that do not induce inflammatory activation. IFNγ stimulates the phagocytosis of $A\beta_{1\text{-}42}$ while at the same time decreasing microglial secretion of BDNF. DHA stimulates phagocytosis and have an inhibitory effect on the secretion of cytokines of both T_{H1} and T_{H2} type.

When studying inflammation in general and inflammation in the CNS in particular, it is apparent that much work remains for the characterization of the different activation phenotypes of macrophage-like cells such as microglia. Considering the detrimental involvement of microglia in neurodegenerative disorders and the potential of microglia to promote neuronal health, an effort to elucidate the response profiles of different activation phenotypes is highly motivated. The author hope that the work presented in this thesis is a contribution to this effort, and he would like to point out a few areas that are associated with AD and which deserves to be investigated:

- The contribution of different surface proteins on human microglia to the recognition and phagocytosis of Aβ

- Beneficial and detrimental microglial responses, aside from phagocytosis, that are consequences of Aβ binding to the surface proteins
- Elucidate the control of the expression of the surface proteins regulating phagocytosis - can they be induced pharmacologically?

A concept that has been touched upon several times in this thesis, and which has just recently been receiving the attention it justly deserves, is the concept of resolution. It may be that resolution has been clouded in obscurity and thus has been regarded as "the thing that happens when inflammation stops and everything is OK again. New research in this field has suggested that resolution is not only the end of inflammation, but an active process that is mediated by molecules that bind to receptors, similar to when inflammation is induced. In fact, it may be futile and contra-productive to even separate the two from each other since resolution is the end-stage of a healthy inflammatory response. To focus exclusively on inhibiting inflammation in therapy may hypothetically leave the tissue in an "unresolved" state, which perhaps is not the most optimal outcome. In fact, an anti-inflammatory role of NF-κB, and the involvement of NF-κB in the resolution of inflammation has been suggested by Lawrence et al [170].

Considering the involvement of inflammation in neurodegenerative disorders, the recent update of the concept of resolution opens up a new area of potential therapeutic targets. In this research, the author believes that some areas should be prioritised:

- Elucidation of the pharmacology of resolution. The receptors that receive pro-resolving signals must be characterised for effective treatments to be developed.
- The link between inflammation and resolution. Is there a transition point which is under the control of factors other than pro-resolving signals?
- Pro-resolving factors in normal conditions. Can they be beneficial for the prevention of neurodegenerative disorders without compromising host immune defence?

6 ACKNOWLEDGEMENTS

My first thank you goes to my main supervisor, Prof. Marianne Schultzberg. You accepted me as a PhD student and introduced me to the fascinating world of neuroscience research. For this I owe you thanks, but most of all for supporting me and letting me (ab)use my academic freedom in a way that I hope has evolved me into something that can be described as a researcher.

I also would like to thank my co-supervisor, Prof. Tomas Hökfelt for accepting me as a student. We did not have much interaction during my work, which I regret, since your knowledge and experience in the field of neuroscience are accepted and respected world-wide.

Next, I would like to thank the members of our group for nice collaboration and support. Heela and Xiuzhe, good luck with your research, you have a long and tough path ahead of you, but I promise it will be lots of fun also. Veronica, thank you for helping me with the flow-cytometry, I hope you soon will analyse samples from your own experiments! Mircea, let us see where headache will take us. Former PhD student in our group also deserves a BIG thank you: Catharina, Stefan and Åsa, it was great working with you, thank you for all your help and support!

A big thank you to all the senior researchers at our facility, for always being helpful and willing to discuss scientific and non-scientific issues. Special thanks to Angel and Helena K for joining in the extremely silly and immature lunch room discussions. Thank you also to Ronnie for nice small-talk and doing the best for keeping the autoclave running…

I had the privilege to spend three months in Boston at Harvard Medical School at the facilities of Dr Howard Weiner. I would like to thank Dr. Weiner for giving me the opportunity to spend three months in Boston at Harvard Medical School where I received new knowledge and experience. Most of this knowledge was channeled to me by Dr. Dan Frenkel. I will never forget your kindness and enthusiasm which really inspired me. I hope to collaborate with you in the future!

Thank you to the former and present Head of Department, Prof. Åke Seiger and Prof. Kerstin Tham, for creating a nice environment for researchers.

Some of the work presented in this thesis is part of the OmegAD study. I would like to thank the Omega-group for involving me in this project. I hope to continue working with you on the research on omega-3 fatty acids. Special thanks to Prof. Jan Palmblad and Dr. Inger Wedin for your invaluable advice on working with fatty acids *in vitro*.

Thank you also to Prof. Bengt Winblad for financial and scientific support through the Swedish Brain Power network.

Flow-cytometry has been a major part of my work in this project and I would like to thank Åsa-Lena Dackland at the Core Facility for much help with understanding and using this technique.

I have met and got to know many PhD-student colleagues and Post-Docs during my PhD-studies. Dear me, without your support and friendship this long journey would not have been as fun and exciting. Thank you for nice conversations, collaboration in the lab, and parties, I will never forget you! There have been a lot of people passing through at this facility during my work here so you are now almost too many to mention, but I would like to give special thanks to a few of you. Liss-Eric and Michi, before you started here there were mostly girls around here, so I must thank you for making me re-discover my manhood and letting it blossom! Anna S, Behnosh, Camilla H, Nodi and Susanne A, we started our projects at almost the same time and therefore it feels like we are almost like "class mates" I will always be thankful for experiencing the harrowing and exciting time which is the start of a PhD-project together with you!

Now the time has come to thank all the non-research people in my life. I feel compelled to start with an apology: forgive me for being absent, boring and uncommunicative during the last five years or so! I promise that I will start a new, more social life now after my dissertation.
I start with thanking my family. Mum, dad, I bet you didn't believe I would be a doctor one day? Anyway, thank you for your love and support, I hope to give some of it back now that life can turn back to normal, somewhat.

My brother and sister, Niclas and Karin, we haven't been able to meet that much during my work since we live in different parts of Sweden, and the life as a PhD student is the way it is. I hope that we can do more things together now, we had a pretty good time in Barcelona, let's go back!

To my non-lab friends I would like to say again that I hope that I will have more time to spend together with you now so that I do not lose even more of you guys…
I would like to thank especially Jenny, for listening to my angry, confused rant and supporting me when things go dark (but as a psychologist you enjoy it, right?).
Thank you also to Erik J, Håkan, Kai and Magnus for winning all my money in poker all the time, and creating a nice diversion from my research in our series of "Friday evening seminars".

Johan B, Patrik (EPA) and Marcus S. Perhaps now that I will have more time, I will make YOU a call for once! Please don't hang up!

Last, I would like to say thank you to the institutions which have provided financial support to my project: Swedish Research Council, Swedish Brain Power, Stiftelsen Forskning utan djurförsök, The Knut and Alice Wallenberg Foundation, Karolinska Institutet Research funds, Stiftelsen för Gamla Tjänarinnor, Alzheimer Foundation Sweden, Gun och Bertil Stohnes stiftelse, and Stockholm County Council.

7 REFERENCES

1. Qiu C, Kivipelto M and von Strauss E (2009) Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. *Dialogues Clin Neurosci* **11:** 111-28
2. Ferri C P, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes P R, Rimmer E and Scazufca M (2005) Global prevalence of dementia: a Delphi consensus study. *Lancet* **366:** 2112-7
3. Selkoe D J (1989) The deposition of amyloid proteins in the aging mammalian brain: implications for Alzheimer's disease. *Ann Med* **21:** 73-6
4. Wolozin B L, Pruchnicki A, Dickson D W and Davies P (1986) A neuronal antigen in the brains of Alzheimer patients. *Science* **232:** 648-50
5. Hardy J and Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol Sci* **12:** 383-8
6. Grundke-Iqbal I, Iqbal K, Tung Y C, Quinlan M, Wisniewski H M and Binder L I (1986) Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A* **83:** 4913-7
7. Selkoe D J, Podlisny M B, Joachim C L, Vickers E A, Lee G, Fritz L C and Oltersdorf T (1988) Beta-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc Natl Acad Sci U S A* **85:** 7341-5
8. Golde T E, Estus S, Younkin L H, Selkoe D J and Younkin S G (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* **255:** 728-30
9. Haass C, Hung A Y, Schlossmacher M G, Teplow D B and Selkoe D J (1993) beta-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *Journal of Biological Chemistry* **268:** 3021-4
10. Nunan J and Small D H (2000) Regulation of APP cleavage by alpha-, beta- and gamma-secretases. *FEBS Lett* **483:** 6-10
11. Octave J N (1995) The amyloid peptide precursor in Alzheimer's disease. *Acta Neurol Belg* **95:** 197-209
12. Kimberly W T, Zheng J B, Guenette S Y and Selkoe D J (2001) The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *Journal of Biological Chemistry* **276:** 40288-92
13. Tamaoka A, Odaka A, Ishibashi Y, Usami M, Sahara N, Suzuki N, Nukina N, Mizusawa H, Shoji S and Kanazawa I (1994) APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. *Journal of Biological Chemistry* **269:** 32721-4
14. Kurt M A, Davies D C, Kidd M, Duff K and Howlett D R (2003) Hyperphosphorylated tau and paired helical filament-like structures in the brains of mice carrying mutant amyloid precursor protein and mutant presenilin-1 transgenes. *Neurobiol Dis* **14:** 89-97
15. Boutajangout A, Authelet M, Blanchard V, Touchet N, Tremp G, Pradier L and Brion J P (2004) Characterisation of cytoskeletal abnormalities in mice transgenic for wild-type human tau and familial Alzheimer's disease mutants of APP and presenilin-1. *Neurobiol Dis* **15:** 47-60
16. Huang H C and Jiang Z F (2009) Accumulated amyloid-beta peptide and hyperphosphorylated tau protein: relationship and links in Alzheimer's disease. *J Alzheimers Dis* **16:** 15-27
17. Lee V M (2001) Biomedicine. Tauists and β-aptists united--well almost! *Science* **293:** 1446-7
18. Takashima A, Noguchi K, Sato K, Hoshino T and Imahori K (1993) Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity. *Proc Natl Acad Sci U S A* **90:** 7789-93
19. Ditaranto K, Tekirian T L and Yang A J (2001) Lysosomal membrane damage in soluble Abeta-mediated cell death in Alzheimer's disease. *Neurobiology of disease* **8:** 19-31

20. Estus S, Tucker H M, van Rooyen C, Wright S, Brigham E F, Wogulis M and Rydel R E (1997) Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant "apoptotic" pattern of gene induction. *J Neurosci* **17:** 7736-45
21. Harada J and Sugimoto M (1999) Activation of caspase-3 in beta-amyloid-induced apoptosis of cultured rat cortical neurons. *Brain Research* **842:** 311-23
22. Butterfield D A, Castegna A, Lauderback C M and Drake J (2002) Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiology of Aging* **23:** 655-64
23. Bezprozvanny I and Mattson M P (2008) Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci* **31:** 454-63
24. Walsh D M, Klyubin I, Fadeeva J V, Cullen W K, Anwyl R, Wolfe M S, Rowan M J and Selkoe D J (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416:** 535-9
25. Xie C W (2004) Calcium-regulated signaling pathways: role in amyloid beta-induced synaptic dysfunction. *Neuromolecular Med* **6:** 53-64
26. Rossner S, Ueberham U, Schliebs R, Perez-Polo J R and Bigl V (1998) The regulation of amyloid precursor protein metabolism by cholinergic mechanisms and neurotrophin receptor signaling. *Prog Neurobiol* **56:** 541-69
27. Wang Y J, Zhou H D and Zhou X F (2006) Clearance of amyloid-beta in Alzheimer's disease: progress, problems and perspectives. *Drug Discov Today* **11:** 931-8
28. Griffin W S, Sheng J G, Roberts G W and Mrak R E (1995) Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. *J Neuropathol Exp Neurol* **54:** 276-81
29. Griffin W S, Stanley L C, Ling C, White L, MacLeod V, Perrot L J, White C L, 3rd and Araoz C (1989) Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *The Proceedings of the National Academy of Sciences* **86:** 7611-5
30. Bauer J, Strauss S, Schreiter-Gasser U, Ganter U, Schlegel P, Witt I, Yolk B and Berger M (1991) Interleukin-6 and alpha-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices. *FEBS Lett* **285:** 111-4
31. McGeer P L, Akiyama H, Itagaki S and McGeer E G (1989) Immune system response in Alzheimer's disease. *Can J Neurol Sci* **16:** 516-27
32. Itagaki S, McGeer P L, Akiyama H, Zhu S and Selkoe D (1989) Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. *J Neuroimmunol* **24:** 173-82
33. Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H and Riederer P (1995) Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neurosci Lett* **202:** 17-20
34. Tarkowski E, Liljeroth A M, Minthon L, Tarkowski A, Wallin A and Blennow K (2003) Cerebral pattern of pro- and anti-inflammatory cytokines in dementias. *Brain Res Bull* **61:** 255-60
35. Ikeda S, Allsop D and Glenner G G (1989) Morphology and distribution of plaque and related deposits in the brains of Alzheimer's disease and control cases. An immunohistochemical study using amyloid beta-protein antibody. *Lab Invest* **60:** 113-22
36. Vercelletto M, Martinez F, Lanier S, Magne C, Jaulin P and Bourin M (2002) Negative symptoms, depression and Alzheimer's disease. *Int J Geriatr Psychiatry* **17:** 383-7
37. Blennow K, de Leon M J and Zetterberg H (2006) Alzheimer's disease. *Lancet* **368:** 387-403
38. Coutureau E and Di Scala G (2009) Entorhinal cortex and cognition. *Prog Neuropsychopharmacol Biol Psychiatry* **33:** 753-61
39. Davies L, Wolska B, Hilbich C, Multhaup G, Martins R, Simms G, Beyreuther K and Masters C L (1988) A4 amyloid protein deposition and the diagnosis of Alzheimer's disease: prevalence in aged brains determined by immunocytochemistry compared with conventional neuropathologic techniques. *Neurology* **38:** 1688-93
40. Rasool C G, Svendsen C N and Selkoe D J (1986) Neurofibrillary degeneration of cholinergic and noncholinergic neurons of the basal forebrain in Alzheimer's disease. *Ann Neurol* **20:** 482-8

41. Reinikainen K J, Riekkinen P J, Paljarvi L, Soininen H, Helkala E L, Jolkkonen J and Laakso M (1988) Cholinergic deficit in Alzheimer's disease: a study based on CSF and autopsy data. *Neurochem Res* **13:** 135-46
42. Lewis P R and Shute C C (1967) The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. *Brain* **90:** 521-40
43. Hedreen J C, Struble R G, Whitehouse P J and Price D L (1984) Topography of the magnocellular basal forebrain system in human brain. *J Neuropathol Exp Neurol* **43:** 1-21
44. Gulyas A I, Acsady L and Freund T F (1999) Structural basis of the cholinergic and serotonergic modulation of GABAergic neurons in the hippocampus. *Neurochem Int* **34:** 359-72
45. Maslinska D and Gajewski M (1998) Some aspects of the inflammatory process. *Folia Neuropathologica* **36:** 199-204
46. Blitstein-Willinger E (1991) The role of growth factors in wound healing. *Skin Pharmacol* **4:** 175-82
47. Bennett N T and Schultz G S (1993) Growth factors and wound healing: Part II. Role in normal and chronic wound healing. *Am J Surg* **166:** 74-81
48. Wahl S M, Wong H and McCartney-Francis N (1989) Role of growth factors in inflammation and repair. *J Cell Biochem* **40:** 193-9
49. Liu K and Nussenzweig M C Origin and development of dendritic cells. *Immunol Rev* **234:** 45-54
50. Auffray C, Sieweke M H and Geissmann F (2009) Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* **27:** 669-92
51. Klein I, Cornejo J C, Polakos N K, John B, Wuensch S A, Topham D J, Pierce R H and Crispe I N (2007) Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. *Blood* **110:** 4077-85
52. Bauer S, Muller T and Hamm S (2009) Pattern recognition by Toll-like receptors. *Adv Exp Med Biol* **653:** 15-34
53. Ozinsky A, Underhill D M, Fontenot J D, Hajjar A M, Smith K D, Wilson C B, Schroeder L and Aderem A (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* **97:** 13766-71
54. Thornalley P J (1998) Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol (Noisy-le-grand)* **44:** 1013-23
55. Husemann J, Loike J D, Anankov R, Febbraio M and Silverstein S C (2002) Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. *Glia* **40:** 195-205
56. Takeuchi O and Akira S Pattern Recognition Receptors and Inflammation. *Cell* **140:** 805-20
57. Bell M D, Lopez-Gonzalez R, Lawson L, Hughes D, Fraser I, Gordon S and Perry V H (1994) Upregulation of the macrophage scavenger receptor in response to different forms of injury in the CNS. *J Neurocytol* **23:** 605-13
58. Chroneos Z and Shepherd V L (1995) Differential regulation of the mannose and SP-A receptors on macrophages. *Am J Physiol* **269:** L721-6
59. Kim Y I, Lee J W, Lee M H, Park S W, Cho B N and Lee H K Effects of 15-deoxy-(12,14)-prostaglandin J (2) on the production of IL-8 and the expression of Toll-like receptor 2 in human primary keratinocytes stimulated with lipopolysaccharide. *Mol Biol Rep*
60. Ihm S H, Chang K, Kim H Y, Baek S H, Youn H J, Seung K B and Kim J H Peroxisome proliferator-activated receptor-gamma activation attenuates cardiac fibrosis in type 2 diabetic rats: the effect of rosiglitazone on myocardial expression of receptor for advanced glycation end products and of connective tissue growth factor. *Basic Res Cardiol* **105:** 399-407
61. Amiel E, Alonso A, Uematsu S, Akira S, Poynter M E and Berwin B (2009) Pivotal Advance: Toll-like receptor regulation of scavenger receptor-A-mediated phagocytosis. *J Leukoc Biol* **85:** 595-605

62. Haslett C, Savill J S, Whyte M K, Stern M, Dransfield I and Meagher L C (1994) Granulocyte apoptosis and the control of inflammation. *Philos Trans R Soc Lond B Biol Sci* **345:** 327-33
63. Ryan A and Godson C Lipoxins: regulators of resolution. *Curr Opin Pharmacol* **10:** 166-72
64. Ohira T, Arita M, Omori K, Recchiuti A, Van Dyke T E and Serhan C N Resolvin E1 receptor activation signals phosphorylation and phagocytosis. *J Biol Chem* **285:** 3451-61
65. Seki H, Tani Y and Arita M (2009) Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1. *Prostaglandins Other Lipid Mediat* **89:** 126-30
66. Anderson P and Delgado M (2008) Endogenous anti-inflammatory neuropeptides and pro-resolving lipid mediators: a new therapeutic approach for immune disorders. *J Cell Mol Med* **12:** 1830-47
67. Schwab J M, Chiang N, Arita M and Serhan C N (2007) Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447:** 869-74
68. Giuliani D, Ottani A, Mioni C, Bazzani C, Galantucci M, Minutoli L, Bitto A, Zaffe D, Botticelli A R, Squadrito F and Guarini S (2007) Neuroprotection in focal cerebral ischemia owing to delayed treatment with melanocortins. *Eur J Pharmacol* **570:** 57-65
69. Ruan L, Kong Y, Wang J M and Le Y Chemoattractants and receptors in Alzheimer's disease. *Front Biosci (Schol Ed)* **2:** 504-14
70. Yedgar S, Cohen Y and Shoseyov D (2006) Control of phospholipase A2 activities for the treatment of inflammatory conditions. *Biochim Biophys Acta* **1761:** 1373-82
71. Khanapure S P, Garvey D S, Janero D R and Letts L G (2007) Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. *Curr Top Med Chem* **7:** 311-40
72. Ke Z J, Calingasan N Y, DeGiorgio L A, Volpe B T and Gibson G E (2005) CD40-CD40L interactions promote neuronal death in a model of neurodegeneration due to mild impairment of oxidative metabolism. *Neurochem Int* **47:** 204-15
73. Chen K, Huang J, Gong W, Zhang L, Yu P and Wang J M (2006) CD40/CD40L dyad in the inflammatory and immune responses in the central nervous system. *Cell Mol Immunol* **3:** 163-9
74. Manning A M, Bell F P, Rosenbloom C L, Chosay J G, Simmons C A, Northrup J L, Shebuski R J, Dunn C J and Anderson D C (1995) NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm* **45:** 283-96
75. Beg A A and Baldwin A S, Jr. (1994) Activation of multiple NF-kappa B/Rel DNA-binding complexes by tumor necrosis factor. *Oncogene* **9:** 1487-92
76. Beg A A, Finco T S, Nantermet P V and Baldwin A S, Jr. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol* **13:** 3301-10
77. O'Dea E and Hoffmann A (2009) NF-kappaB signaling. *Wiley Interdiscip Rev Syst Biol Med* **1:** 107
78. Briscoe J, Guschin D, Rogers N C, Watling D, Muller M, Horn F, Heinrich P, Stark G R and Kerr I M (1996) JAKs, STATs and signal transduction in response to the interferons and other cytokines. *Philos Trans R Soc Lond B Biol Sci* **351:** 167-71
79. Darnell J E, Jr., Kerr I M and Stark G R (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264:** 1415-21
80. Vila-del Sol V, Diaz-Munoz M D and Fresno M (2007) Requirement of tumor necrosis factor alpha and nuclear factor-kappaB in the induction by IFN-gamma of inducible nitric oxide synthase in macrophages. *J Leukoc Biol* **81:** 272-83
81. Mir M, Tolosa L, Asensio V J, Llado J and Olmos G (2008) Complementary roles of tumor necrosis factor alpha and interferon gamma in inducible microglial nitric oxide generation. *J Neuroimmunol* **204:** 101-9
82. Sekine N, Ishikawa T, Okazaki T, Hayashi M, Wollheim C B and Fujita T (2000) Synergistic activation of NF-kappab and inducible isoform of nitric oxide synthase induction by interferon-gamma and tumor necrosis factor-alpha in INS-1 cells. *J Cell Physiol* **184:** 46-57

83. Zhang F, Liu F, Yan M, Ji H, Hu L, Li X, Qian J, He X, Zhang L, Shen A and Cheng C Peroxisome proliferator-activated receptor-gamma agonists suppress iNOS expression induced by LPS in rat primary Schwann cells. *J Neuroimmunol* **218:** 36-47
84. Kalinin S, Richardson J C and Feinstein D L (2009) A PPARdelta agonist reduces amyloid burden and brain inflammation in a transgenic mouse model of Alzheimer's disease. *Curr Alzheimer Res* **6:** 431-7
85. Chen H H, Chen T W and Lin H (2009) Prostacyclin-induced peroxisome proliferator-activated receptor-alpha translocation attenuates NF-kappaB and TNF-alpha activation after renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* **297:** F1109-18
86. Adibhatla R M and Hatcher J F (2008) Phospholipase A(2), reactive oxygen species, and lipid peroxidation in CNS pathologies. *BMB Rep* **41:** 560-7
87. Downen M, Amaral T D, Hua L L, Zhao M L and Lee S C (1999) Neuronal death in cytokine-activated primary human brain cell culture: role of tumor necrosis factor-alpha. *Glia* **28:** 114-27
88. Butler M P, O'Connor J J and Moynagh P N (2004) Dissection of tumor-necrosis factor-alpha inhibition of long-term potentiation (LTP) reveals a p38 mitogen-activated protein kinase-dependent mechanism which maps to early-but not late-phase LTP. *Neuroscience* **124:** 319-26
89. Vereker E, O'Donnell E, Lynch A, Kelly A, Nolan Y and Lynch M A (2001) Evidence that interleukin-1beta and reactive oxygen species production play a pivotal role in stress-induced impairment of LTP in the rat dentate gyrus. *Eur J Neurosci* **14:** 1809-19
90. Curran B P, Murray H J and O'Connor J J (2003) A role for c-Jun N-terminal kinase in the inhibition of long-term potentiation by interleukin-1beta and long-term depression in the rat dentate gyrus in vitro. *Neuroscience* **118:** 347-57
91. Choi D W (1992) Excitotoxic cell death. *J Neurobiol* **23:** 1261-76
92. Shaw P J (1992) Excitatory amino acid neurotransmission, excitotoxicity and excitotoxins. *Curr Opin Neurol Neurosurg* **5:** 383-90
93. Obrenovitch T P (2001) Quinolinic acid accumulation during neuroinflammation. Does it imply excitotoxicity? *Ann N Y Acad Sci* **939:** 1-10
94. Guillemin G J and Brew B J (2002) Implications of the kynurenine pathway and quinolinic acid in Alzheimer's disease. *Redox Rep* **7:** 199-206
95. Braidy N, Grant R, Adams S, Brew B J and Guillemin G J (2009) Mechanism for quinolinic acid cytotoxicity in human astrocytes and neurons. *Neurotox Res* **16:** 77-86
96. Sturrock R R (1988) The beta astrocyte: its possible role as a central nervous system phagocyte. *Anat Anz* **166:** 331-40
97. Kakita H, Aoyama M, Hussein M H, Kato S, Suzuki S, Ito T, Togari H and Asai K (2009) Diclofenac enhances proinflammatory cytokine-induced nitric oxide production through NF-kappaB signaling in cultured astrocytes. *Toxicol Appl Pharmacol* **238:** 56-63
98. Cardona A E, Pioro E P, Sasse M E, Kostenko V, Cardona S M, Dijkstra I M, Huang D, Kidd G, Dombrowski S, Dutta R, Lee J C, Cook D N, Jung S, Lira S A, Littman D R and Ransohoff R M (2006) Control of microglial neurotoxicity by the fractalkine receptor. *Nat Neurosci* **9:** 917-24
99. Hatori K, Nagai A, Heisel R, Ryu J K and Kim S U (2002) Fractalkine and fractalkine receptors in human neurons and glial cells. *J Neurosci Res* **69:** 418-26
100. Mizoguchi Y, Monji A, Kato T, Seki Y, Gotoh L, Horikawa H, Suzuki S O, Iwaki T, Yonaha M, Hashioka S and Kanba S (2009) Brain-derived neurotrophic factor induces sustained elevation of intracellular Ca2+ in rodent microglia. *J Immunol* **183:** 7778-86
101. Asami T, Ito T, Fukumitsu H, Nomoto H, Furukawa Y and Furukawa S (2006) Autocrine activation of cultured macrophages by brain-derived neurotrophic factor. *Biochem Biophys Res Commun* **344:** 941-7
102. Mu J S, Li W P, Yao Z B and Zhou X F (1999) Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Res* **835:** 259-65
103. Mitchell J J, Paiva M, Walker D W and Heaton M B (1999) BDNF and NGF afford in vitro neuroprotection against ethanol combined with acute ischemia and chronic hypoglycemia. *Dev Neurosci* **21:** 68-75

104. Nakanishi M, Niidome T, Matsuda S, Akaike A, Kihara T and Sugimoto H (2007) Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells. *Eur J Neurosci* **25:** 649-58
105. Beck T, Lindholm D, Castren E and Wree A (1994) Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. *J Cereb Blood Flow Metab* **14:** 689-92
106. Harper M M, Adamson L, Blits B, Bunge M B, Grozdanic S D and Sakaguchi D S (2009) Brain-derived neurotrophic factor released from engineered mesenchymal stem cells attenuates glutamate- and hydrogen peroxide-mediated death of staurosporine-differentiated RGC-5 cells. *Exp Eye Res* **89:** 538-48
107. Yamada K, Mizuno M and Nabeshima T (2002) Role for brain-derived neurotrophic factor in learning and memory. *Life Sci* **70:** 735-44
108. Lu B and Gottschalk W (2000) Modulation of hippocampal synaptic transmission and plasticity by neurotrophins. *Prog Brain Res* **128:** 231-41
109. Black I B (1999) Trophic regulation of synaptic plasticity. *J Neurobiol* **41:** 108-18
110. Nakajima K, Honda S, Tohyama Y, Imai Y, Kohsaka S and Kurihara T (2001) Neurotrophin secretion from cultured microglia. *J Neurosci Res* **65:** 322-31
111. McDonald D R, Bamberger M E, Combs C K and Landreth G E (1998) beta-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. *J Neurosci* **18:** 4451-60
112. Akama K T, Albanese C, Pestell R G and Van Eldik L J (1998) Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. *Proc Natl Acad Sci U S A* **95:** 5795-800
113. Meda L, Baron P and Scarlato G (2001) Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins. *Neurobiol Aging* **22:** 885-93
114. Bamberger M E and Landreth G E (2001) Microglial interaction with beta-amyloid: implications for the pathogenesis of Alzheimer's disease. *Microsc Res Tech* **54:** 59-70
115. Dash P K and Moore A N (1995) Enhanced processing of APP induced by IL-1 beta can be reduced by indomethacin and nordihydroguaiaretic acid. *Biochem Biophys Res Commun* **208:** 542-8
116. Sommer G, Kralisch S, Lipfert J, Weise S, Krause K, Jessnitzer B, Lossner U, Bluher M, Stumvoll M and Fasshauer M (2009) Amyloid precursor protein expression is induced by tumor necrosis factor alpha in 3T3-L1 adipocytes. *J Cell Biochem* **108:** 1418-22
117. Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P and Grubeck-Loebenstein B (2000) Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ by human astrocytes. *Neurobiological Disorders* **7:** 682-9
118. Jensen M T, Mottin M D, Cracchiolo J R, Leighty R E and Arendash G W (2005) Lifelong immunization with human beta-amyloid (1-42) protects Alzheimer's transgenic mice against cognitive impairment throughout aging. *Neuroscience* **130:** 667-84
119. Janabi N, Peudenier S, Heron B, Ng K H and Tardieu M (1995) Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neuroscience Letters* **195:** 105-8
120. Bryan T M and Reddel R R (1994) SV40-induced immortalization of human cells. *Crit Rev Oncog* **5:** 331-57
121. Pahlman S, Ruusala A I, Abrahamsson L, Mattsson M E and Esscher T (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differ* **14:** 135-44
122. Kaplan D R, Matsumoto K, Lucarelli E and Thiele C J (1993) Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group. *Neuron* **11:** 321-31
123. Frenkel D, Puckett L, Petrovic S, Xia W, Chen G, Vega J, Dembinsky-Vaknin A, Shen J, Plante M, Burt D S and Weiner H L (2008) A nasal proteosome adjuvant activates microglia and prevents amyloid deposition. *Annals of Neurology* **63:** 591-601
124. Bluthe R M, Crestani F, Kelley K W and Dantzer R (1992) Mechanisms of the behavioral effects of interleukin 1. Role of prostaglandins and CRF. *Ann N Y Acad Sci* **650:** 268-75

125. Giulian D, Young D G, Woodward J, Brown D C and Lachman L B (1988) Interleukin-1 is an astroglial growth factor in the developing brain. *J Neurosci* **8:** 709-14
126. Bazan N G (2009) Cellular and molecular events mediated by docosahexaenoic acid-derived neuroprotectin D1 signaling in photoreceptor cell survival and brain protection. *Prostaglandins Leukot Essent Fatty Acids* **81:** 205-11
127. Serhan C N, Yang R, Martinod K, Kasuga K, Pillai P S, Porter T F, Oh S F and Spite M (2009) Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med* **206:** 15-23
128. Li H, Ruan X Z, Powis S H, Fernando R, Mon W Y, Wheeler D C, Moorhead J F and Varghese Z (2005) EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. *Kidney Int* **67:** 867-74
129. Forslin Aronsson S, Spulber S, Popescu L M, Winblad B, Post C, Oprica M and Schultzberg M (2006) alpha-Melanocyte-stimulating hormone is neuroprotective in rat global cerebral ischemia. *Neuropeptides* **40:** 65-75
130. Hadley M E, Hruby V J, Jiang J, Sharma S D, Fink J L, Haskell-Luevano C, Bentley D L, al-Obeidi F and Sawyer T K (1996) Melanocortin receptors: identification and characterization by melanotropic peptide agonists and antagonists. *Pigment Cell Res* **9:** 213-34
131. Brzoska T, Luger T A, Maaser C, Abels C and Bohm M (2008) Alpha-melanocyte-stimulating hormone and related tripeptides: biochemistry, antiinflammatory and protective effects in vitro and in vivo, and future perspectives for the treatment of immune-mediated inflammatory diseases. *Endocr Rev* **29:** 581-602
132. Catania A (2008) Neuroprotective actions of melanocortins: a therapeutic opportunity. *Trends Neurosci* **31:** 353-60
133. Lasaga M, Debeljuk L, Durand D, Scimonelli T N and Caruso C (2008) Role of alpha-melanocyte stimulating hormone and melanocortin 4 receptor in brain inflammation. *Peptides* **29:** 1825-35
134. Lindberg C, Selenica M L, Westlind-Danielsson A and Schultzberg M (2005) β-amyloid protein structure determines the nature of cytokine release from rat microglia. *Journal of Molecular Neuroscience* **27:** 1-12
135. Necula M, Breydo L, Milton S, Kayed R, van der Veer W E, Tone P and Glabe C G (2007) Methylene blue inhibits amyloid Abeta oligomerization by promoting fibrillization. *Biochemistry* **46:** 8850-60
136. Sokolova A, Hill M D, Rahimi F, Warden L A, Halliday G M and Shepherd C E (2009) Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease. *Brain Pathol* **19:** 392-8
137. Zorina Y, Iyengar R and Bromberg K D Cannabinoid 1 receptor and interleukin-6 receptor together induce integration of protein kinase and transcription factor signaling to trigger neurite outgrowth. *J Biol Chem* **285:** 1358-70
138. Schafer K H, Mestres P, Marz P and Rose-John S (1999) The IL-6/sIL-6R fusion protein hyper-IL-6 promotes neurite outgrowth and neuron survival in cultured enteric neurons. *J Interferon Cytokine Res* **19:** 527-32
139. Wu Y Y and Bradshaw R A (1996) Induction of neurite outgrowth by interleukin-6 is accompanied by activation of Stat3 signaling pathway in a variant PC12 cell (E2) line. *J Biol Chem* **271:** 13023-32
140. Matsuda S, Wen T C, Morita F, Otsuka H, Igase K, Yoshimura H and Sakanaka M (1996) Interleukin-6 prevents ischemia-induced learning disability and neuronal and synaptic loss in gerbils. *Neurosci Lett* **204:** 109-12
141. Qiu Z and Gruol D L (2003) Interleukin-6, beta-amyloid peptide and NMDA interactions in rat cortical neurons. *J Neuroimmunol* **139:** 51-7
142. Taga T and Fukuda S (2005) Role of IL-6 in the neural stem cell differentiation. *Clin Rev Allergy Immunol* **28:** 249-56
143. Marz P, Heese K, Dimitriades-Schmutz B, Rose-John S and Otten U (1999) Role of interleukin-6 and soluble IL-6 receptor in region-specific induction of astrocytic differentiation and neurotrophin expression. *Glia* **26:** 191-200
144. Bartfai T, Sanchez-Alavez M, Andell-Jonsson S, Schultzberg M, Vezzani A, Danielsson E and Conti B (2007) Interleukin-1 system in CNS stress: seizures, fever, and neurotrauma. *Ann N Y Acad Sci* **1113:** 173-7

145. Tong L, Thornton P L, Balazs R and Cotman C W (2001) Beta -amyloid-(1-42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival Is not compromised. *J Biol Chem* **276:** 17301-6
146. Tong L, Balazs R, Thornton P L and Cotman C W (2004) Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons. *J Neurosci* **24:** 6799-809
147. Shirshev S V and Orlova E G (2005) Molecular mechanisms of regulation of functional activity of mononuclear phagocytes by leptin. *Biochemistry (Mosc)* **70:** 841-7
148. Virelizier J L and Arenzana-Seisdedos F (1985) Immunological functions of macrophages and their regulation by interferons. *Med Biol* **63:** 149-59
149. Lee J S, Yang C S, Shin D M, Yuk J M, Son J W and Jo E K (2009) Nitric Oxide Synthesis is Modulated by 1,25-Dihydroxyvitamin D3 and Interferon-gamma in Human Macrophages after Mycobacterial Infection. *Immune Netw* **9:** 192-202
150. Xie Q W, Whisnant R and Nathan C (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med* **177:** 1779-84
151. Koide M, Kawahara Y, Tsuda T and Yokoyama M (1993) Cytokine-induced expression of an inducible type of nitric oxide synthase gene in cultured vascular smooth muscle cells. *FEBS Lett* **318:** 213-7
152. Basham T, Smith W, Lanier L, Morhenn V and Merigan T (1984) Regulation of expression of class II major histocompatibility antigens on human peripheral blood monocytes and Langerhans cells by interferon. *Hum Immunol* **10:** 83-93
153. Boivin A, Pineau I, Barrette B, Filali M, Vallieres N, Rivest S and Lacroix S (2007) Toll-like receptor signaling is critical for Wallerian degeneration and functional recovery after peripheral nerve injury. *J Neurosci* **27:** 12565-76
154. Ribes S, Ebert S, Regen T, Agarwal A, Tauber S C, Czesnik D, Spreer A, Bunkowski S, Eiffert H, Hanisch U K, Hammerschmidt S and Nau R Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. *Infect Immun* **78:** 865-71
155. Li W, Gao G, Guo Q, Jia D, Wang J, Wang X, He S and Liang Q (2009) Function and phenotype of microglia are determined by toll-like receptor 2/toll-like receptor 4 activation sequence. *DNA Cell Biol* **28:** 493-9
156. Reed-Geaghan E G, Savage J C, Hise A G and Landreth G E (2009) CD14 and toll-like receptors 2 and 4 are required for fibrillar A{beta}-stimulated microglial activation. *J Neurosci* **29:** 11982-92
157. Paradisi S, Matteucci A, Fabrizi C, Denti M A, Abeti R, Breit S N, Malchiodi-Albedi F and Mazzanti M (2008) Blockade of chloride intracellular ion channel 1 stimulates Abeta phagocytosis. *J Neurosci Res* **86:** 2488-98
158. Nagano T, Kimura S H and Takemura M Prostaglandin E(2) reduces amyloid beta-induced phagocytosis in cultured rat microglia. *Brain Res*
159. Familian A, Eikelenboom P and Veerhuis R (2007) Minocycline does not affect amyloid beta phagocytosis by human microglial cells. *Neurosci Lett* **416:** 87-91
160. Pober J S, Slowik M R, De Luca L G and Ritchie A J (1993) Elevated cyclic AMP inhibits endothelial cell synthesis and expression of TNF-induced endothelial leukocyte adhesion molecule-1, and vascular cell adhesion molecule-1, but not intercellular adhesion molecule-1. *J Immunol* **150:** 5114-23
161. Aloisi F, De Simone R, Columba-Cabezas S and Levi G (1999) Opposite effects of interferon-gamma and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. *J Neurosci Res* **56:** 571-80
162. Wachira S J, Hughes-Darden C A, Taylor C V, Ochillo R and Robinson T J (2003) Evidence for the interaction of protein kinase C and melanocortin 3-receptor signaling pathways. *Neuropeptides* **37:** 201-10
163. Chai B, Li J Y, Zhang W, Ammori J B and Mulholland M W (2007) Melanocortin-3 receptor activates MAP kinase via PI3 kinase. *Regul Pept* **139:** 115-21
164. Rodrigues A R, Pignatelli D, Almeida H and Gouveia A M (2009) Melanocortin 5 receptor activates ERK1/2 through a PI3K-regulated signaling mechanism. *Mol Cell Endocrinol* **303:** 74-81

165. Vongs A, Lynn N M and Rosenblum C I (2004) Activation of MAP kinase by MC4-R through PI3 kinase. *Regul Pept* **120:** 113-8
166. Sizemore N, Leung S and Stark G R (1999) Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol Cell Biol* **19:** 4798-805
167. Dinarello C A (1998) Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *Int Rev Immunol* **16:** 457-99
168. Dickensheets H L and Donnelly R P (1997) IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. *J Immunol* **159:** 6226-33
169. Garlind A, Brauner A, Hojeberg B, Basun H and Schultzberg M (1999) Soluble interleukin-1 receptor type II levels are elevated in cerebrospinal fluid in Alzheimer's disease patients. *Brain Res* **826:** 112-6
170. Lawrence T and Fong C The resolution of inflammation: anti-inflammatory roles for NF-kappaB. *Int J Biochem Cell Biol* **42:** 519-23

I

Neurotoxicity Research, 2005, VOL. 8(3,4). pp. 267-276

F.P. Graham Publishing Co.

Cytokine Production by a Human Microglial Cell Line: Effects of β-Amyloid and α-Melanocyte-Stimulating Hormone

CATHARINA LINDBERG[a], ERIK HJORTH[a], CLAES POST[b], BENGT WINBLAD[a] and MARIANNE SCHULTZBERG[a,*]

[a]*Karolinska Institutet, Neurotec Department, Division of Experimental Geriatrics, Karolinska University Hospital Huddinge, Novum, SE-141 86 Stockholm, Sweden;* [b]*Gastrotech Pharma, Nyhavn 43B, DK-1051, Copenhagen K, Denmark. Marianne.Schultzberg@ki.se*

(Received 16 May 2005; Revised 17 August 2005; In final form 17 August 2005)

Senile plaques in the Alzheimer's disease (AD) are formed by aggregation of β-amyloid (Aβ) peptide. Aβ peptide has been shown to activate microglia and stimulate their production of inflammatory factors, such as cytokines. In the AD brain, the continued presence of amyloid plaques may keep microglia persistently activated, leading to chronic inflammation in the CNS. It is well established that α-melanocyte-stimulating hormone (α-MSH) gives rise to anti-inflammatory and anti-pyretic effects. The biological activities of α-MSH are mediated by one or more of the melanocortin receptor (MCR) subtypes, *i.e.,* MCR1 - MCR5. The aim of the present study was to determine the effect of α-MSH alone and on Aβ-activated microglial cells with regard to the secretion of inflammatory cytokines, such as interleukin-6 (IL-6), and to determine which receptor subtype mediates the effects of α-MSH. The human microglial cell line, CHME3, was incubated for 24 h with freshly dissolved $A\beta_{1\text{-}40}$, interferon-γ (IFN-γ) and/or α-MSH. Freshly dissolved $A\beta_{1\text{-}40}$ (5-60 μM) resulted in a dose-dependent decrease in cell viability, along with a dose-dependent increase in IL-6 release. Neither IFN-γ nor α-MSH affected the Aβ-induced secretion of IL-6, but resulted in a dose-dependent increase in basal IL-6 release. Agouti, the endogenous antagonist of MCR1 and 4, further increased the α-MSH-induced secretion of IL-6. RT-PCR showed the expression of MCR1, MCR3, MCR4 and MCR5 mRNA. The combined data suggest that the effect of α-MSH in increasing IL-6 release from the human microglial cell line is mediated by MCR3 or MCR5.

Keywords: Alzheimer's disease; Cell viability; Interferon; Interleukin; Melanocortin receptors

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the elderly. The senile plaques in AD are formed by aggregation of β-amyloid (Aβ) peptide, a 39-42 amino acid cleavage product of β-amyloid precursor protein (APP). Senile plaques in the AD brain are surrounded by activated astrocytes and microglia, the source of inflammatory and cytotoxic factors. The reactive glial cells produce cytokines such as interleukin-1 (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (Griffin *et al.*, 1989; Bauer *et al.*, 1991; Dickson *et al.*, 1993), as well as chemokines, complement factors and acute phase reactants (Vandenabeele and Fiers, 1991; Eikelenboom *et al.*, 1994; Neuroinflammation working group, 2000; Galimberti *et al.*, 2003; McGeer and McGeer, 2003). Furthermore, elevated levels of IL-1β (Cacabelos *et al.*, 1991; Blum-Degen *et al.*, 1995), TNF-α (Tarkowski *et al.*, 1999) and chemokines (Galimberti *et al.*, 2003), have been observed in serum and cerebrospinal fluid (CSF) from AD-patients, whereas the levels of IL-6 have been shown to be either increased or unaltered as compared to healthy, age-matched controls (Blum-Degen *et al.*, 1995; Hampel *et al.*, 1997; Martinez *et al.*, 2000; Neuroinflammation working group, 2000). Significant activation of microglia appears to occur at an early stage of the disease, *i.e.*, before severe cognitive decline has occurred (Vehmas *et al.*, 2003).

There is ample evidence that Aβ peptide stimulates the activation of microglia, leading to increased synthesis and release of pro-inflammatory cytokines (Araujo and Cotman, 1992; Del Bo *et al.*, 1995; Lindberg *et al.*, 2005), as well as cytotoxic factors such as proteolytic enzymes, nitric oxide (NO), quinolinic acid, glutamate,

*Corresponding author. Tel.: +46 8 585-83889; FAX: +46 8 585-83850; E-mail: Marianne.Schultzberg@ki.se
ISSN 1029 8428 print/ ISSN 1476-3524 online.

and reactive oxygen species (ROS) (see Benveniste *et al.*, 2001). Some of these microglial products may be responsible for the indirect neurotoxic effects of Aβ (Selkoe, 1994; Giulian *et al.*, 1996).

It is well established that α-melanocyte-stimulating hormone (α-MSH) gives rise to anti-inflammatory (see Catania and Lipton, 1998) and anti-pyretic effects (Glyn and Lipton, 1981). For example, α-MSH has been shown to inhibit hyperalgesia induced by the pro-inflammatory cytokine IL-1β in rats (Oka *et al.*, 1996). The biological activities of α-MSH are mediated by one or more of the melanocortin receptor (MCR) subtypes, *i.e.*, MCR 1 - MCR 5 (see Tatro, 1996; Adan and Gispen, 1997; Wikberg, 1999; Cone, 2005). The most abundant subtypes in the brain are MCR3 and MCR 4, whereas the expression of MCR5 is lower. MCR1 is mainly expressed in monocytes (Rajora *et al.*, 1996), and MCR2 mainly in the adrenal cortex (Mountjoy *et al.*, 1992; Cammas *et al.*, 1995).

The aims of this study were to investigate the effects of $A\beta_{1-40}$ on a human microglial cell line with regard to the secretion of cytokines and cell viability. The possibility to modulate the microglial responses to $A\beta_{1-40}$ with α-MSH was analysed, as well as the effects of α-MSH on microglia under basal conditions.

METHODS

Materials

Human $A\beta_{1-40}$, in a lyophilised trifluoroacetate (TFA) salt, was purchased from rPeptide (Athens, GA, USA). α-MSH was obtained from Melacure AB (Uppsala, Sweden) or Bachem Holding AG (Germany), and agouti from Phoenix Pharmaceuticals, Inc (Belmont, USA). Interferon-γ (IFN-γ), 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT), H89, dimethyl sulfoxide (DMSO), and GeneElute™ Mammalian Total RNA Miniprep Kit, were obtained from Sigma-Aldrich, Inc. (Stockholm, Sweden). IL-6 Quantikine Duoset, TNF-α and IL-1β high sensitivity (HS) kits, and IL-1α and caspase-1 ELISA kits were obtained from R&D Systems Europe, Ltd (Oxon, UK). Dulbecco's modified Eagle medium (DMEM) with high glucose, foetal bovine serum (FBS), gentamicin, L-glutamine, TryPLE and Taq-platinum were purchased from Invitrogen, Life technologies (Stockholm, Sweden). RQ1 RNase-free DNase, AMV Reverse Transcription System and Nuclease-free dH_2O were purchased from SDS Biosciences (Falkenberg, Sweden). All other organic reagents were purchased from VWR International AB (Stockholm, Sweden).

Cell Cultures

The CHME-3 microglial cell line was a kind gift from Professor M. Tardieu (Université Paris Sud, France). Human primary microglia from 8 - 10 week embryos were immortalised by transfection with the SV40 large T antigen (Janabi *et al.*, 1995). The cells were grown in DMEM-containing high glucose (4.5 g/l), L-glutamine, 10% foetal calf serum and gentamicin, and maintained at 37°C in a humidified atmosphere with 5% CO_2. The CHME-3 cells were tested for and free of mycoplasmic infection.

Cell Stimulation

CHME-3 cells were seeded into 24-well plates at a concentration of 2.5 x 10^4 cells per well and cultured in serum-containing medium for 24 h. In order to treat the microglia, the culture medium was removed and replaced by fresh medium without serum and antibiotics. A dose-response curve with 5-60 μM freshly dissolved $A\beta_{1-40}$ was performed. The concentrations of 20 and 40 μM $A\beta_{1-40}$ were used for a time-response curve between 5 and 48 h. Dose-response curves of 1 - 100 μM IFN-γ, 0.001 - 20 μM α-MSH and 0.1- 20 μM agouti and combination treatments were performed for 24 h. The $A\beta_{1-40}$ peptide was dissolved in distilled H_2O and then diluted in medium immediately prior to use. The IFN-γ, agouti and H89 were dissolved in serum-free medium, and α-MSH was dissolved in saline, prior to incubation with the microglial cells. Co-stimulation with Aβ and IFN-γ were performed in the presence or absence of α-MSH. The stimulation with α-MSH was also performed in the presence of agouti or the protein kinase A (PKA) inhibitor H89. Each condition was carried out in 4 wells (3 for treatment with agouti) and each type of treatment was performed in 3 independent experiments (4 for IFN-γ).

Measurements of Cytokine Secretion

The culture medium was collected after 24 h of incubation, or at the time points 5, 8, 24, 30 and 48h for the time-response curve, snap frozen in liquid nitrogen and stored at -70°C until use. All samples were centrifuged before analysis. Secretion of IL-6, TNF-α, IL-1α, IL-1β and caspase-1 to the culture medium was assessed by commercially available ELISA-kits, according to the manufacturer's protocols. For each set of experiments, standard curves were run. The secretion of IL-6 after treatment with the different substances was normalised against the basal secretion, *i.e.*, after vehicle treatment.

Cell Viability

The cell viability was analysed using the colometric

MTT assay. MTT was dissolved in serum-free culture medium to a final concentration of 0.3 mg/ml and then added to the cells for 1 h at 37°C. The medium was then aspirated and the insoluble product remaining in the culture well was dissolved in DMSO. The resulting solutions were analysed in duplicates in a spectrophotometer at 592 nm, the absorbance maximum for the coloured product of reduced MTT, formed by the activity of mitochondrial dehydrogenases in viable cells. The OD values of the treated cells were normalised against the controls.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to analyse the expression of MCR subtypes in the CHME-3 cells. Cells from six different passages in six independent experiments were detached with TryPLE, centrifuged for 5 min at 1000*g* in a Wifug centrifuge and the pellets were then washed with phosphate-buffered saline (PBS). The pellets were dissolved with the lysis solution supplied with the GeneElute^{TM} Mammalian Total RNA Miniprep Kit and RNA extraction was performed according to the manufacturer's protocol. The resulting RNA extracts were then digested with RQ1 RNase-free DNase. First strand synthesis was performed using AMV Reverse Transcription System and the resulting cDNA was amplified with Taq-platinum in an Eppendorff Mastercycler Gradient PCR machine with the following program: (1) 94°C 5 min; (2) 94°C 30 sec; (3) X°C

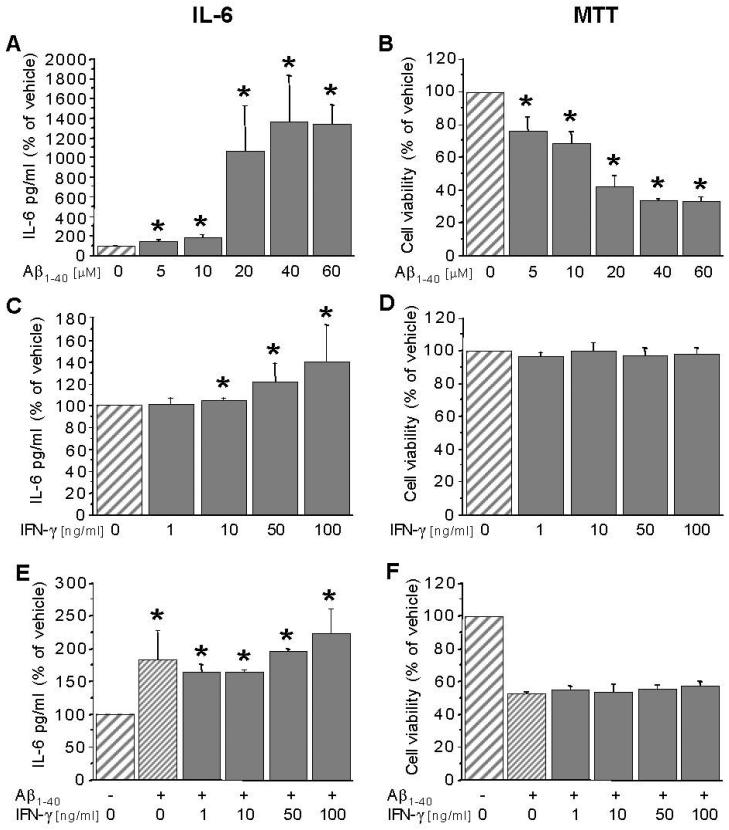

FIGURE 1 A-F Effects on the release of IL-6 (**A,C,E**) and cell viability (**B,D,F**) in cultures of the human microglial cell line CHME-3 exposed to $A\beta_{1-40}$ (**A,B**), IFN-γ (**C,D**) or 10 μM Aβ + IFN-γ (**E,F**). The amount of cytokine release and cell viability after treatment is expressed as % of vehicle, which is set to 100%. Vehicle (indicated by bars with thick stripes) = 0 μM $A\beta_{1-40}$ (**A,B**), 0 μM IFN-γ (**C,D**), and 0 μM $A\beta_{1-40}$ + 0 μM IFN-γ (**E,F**). Error bars represent standard deviation. Statistical significance is defined as $p < 0.05$ as compared to vehicle (indicated by *). The non-parametric Kruskal-Wallis test and the Mann-Whitney test were used for statistical analyses. $A\beta_{1-40}$ = β-amyloid; IFN-γ = interferon-γ; IL-6 = interleukin-6; MTT = 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide.

1 min; (4) 72°C 1 min (the (2) - (4) steps were repeated with X being 63°C for the first cycle and 58°C for the last cycle with decrements of 0.5°C in between); (5) 94°C 30 sec; (6) 58°C 1 min; (7) 72°C 1 min ((5) - (7) were repeated 25 times); (8) 72°C 7 min; (9) 4°C (infinity). The primer sequences used were the same as those previously reported by Böhm *et al.* (Böhm *et al.*, 2002). The primers were used in a concentration of 0.02 µM.

Positive controls (genomic DNA extracted from the human microglial cell line) and negative controls (DNase-digested RNA-extract not subjected to reverse transcription) were included in all reactions. The PCR-products were analysed on a 1.5% agarose gel stained with 10 µg/ml ethidium bromide. The results were documented with a Fujifilm Diana 1 CCD-camera connected to a computer with Diana 1.6 software.

Statistical Analysis

Data were analysed with the non-parametric Kruskal-Wallis test. The Mann-Whitney test was used for the statistical comparisons between groups. The statistical significance was established at a level of $p < 0.05$.

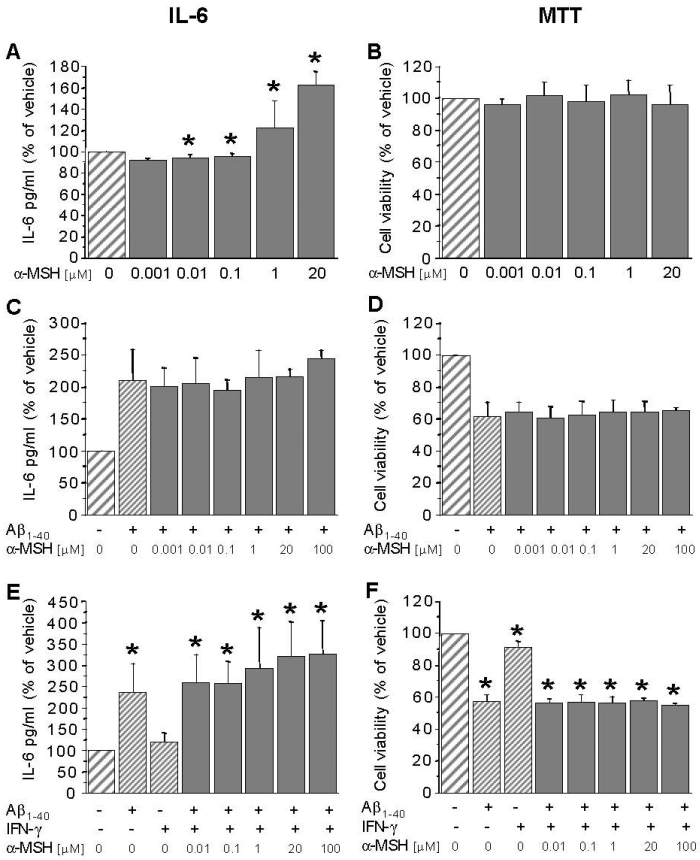

FIGURE 2 A-F Effects on the release of IL-6 (**A,C,E**) and cell viability (**B,D,F**) in cultures of the human microglial cell line CHME-3 exposed to α-MSH (**A,B**), 10 µM $A\beta_{1-40}$ + α-MSH (**C,D**) or 10 µM $A\beta_{1-40}$ + 100 ng/ml IFN-γ + α-MSH (E,F). The amount of cytokine release and cell viability after treatment is expressed as % of vehicle, which is set to 100%. Vehicle (indicated by bars with thick stripes) = 0 µM α-MSH (**A,B**), 0 µM $A\beta_{1-40}$ + 0 µM α-MSH (**C,D**) and 0 µM $A\beta_{1-40}$ + 0 µM IFN-γ (**E,F**). Error bars represent standard deviation. Statistical significance is defined as $p < 0.05$ as compared to vehicle (indicated by *). The non-parametric Kruskal-Wallis test and the Mann-Whitney test were used for statistical analyses. $A\beta_{1-40}$ = β-amyloid$_{1-40}$; α-MSH = α-melanocyte-stimulating hormone; IFN-γ = interferon- γ; IL-6 = interleukin-6; MTT = 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide.

RESULTS

The effects of α-MSH on the secretion of IL-6 and on cellular viability were assessed in a human microglial cell line, CHME3, both under basal conditions and in cells activated with $A\beta_{1-40}$, and/or the inflammatory cytokine IFN-γ. In addition, the cellular expression of different receptors for α-MSH, MCRs, was analysed.

Cytokine Secretion

The time-response curve for $A\beta_{1-40}$ showed a small increase in the secretion of IL-6 from the human microglia already after 5 h incubation with 20 or 40 μM $A\beta_{1-40}$, with a continuous increase up to 48 h (data not shown). Incubation of the cells with $A\beta_{1-40}$ at 10, 20, 40 or 60 μM or with vehicle, showed a very low, or non-detectable, secretion of TNF-α, IL-1α, IL-1β and caspase-1 (data not shown), and these proteins were not analysed further.

Activation of the microglia with freshly dissolved $A\beta_{1-40}$ (FIG. 1A) or the incubation with IFN-γ (FIG. 1C), resulted in a dose-dependent increase in IL-6 secretion as compared to basal secretion.

The dose of 10 μM $A\beta_{1-40}$ resulted in about 100%

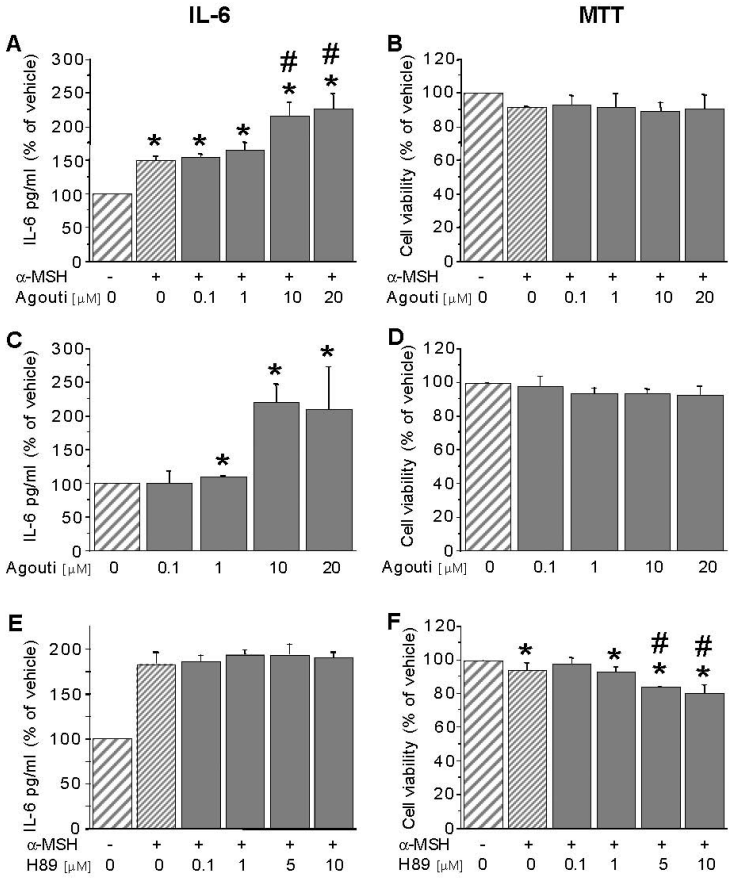

FIGURE 3 A-F Effects on the release of IL-6 (**A,C,E**) and cell viability (**B,D,F**) in cultures of the human microglial cell line CHME-3 exposed to 20 μM α-MSH + agouti (**A,B**), agouti (**C,D**) or 20 μM α-MSH + H89 (**E,F**). The amount of cytokine release and cell viability after treatment is expressed as % of vehicle, which is set to 100%. Vehicle (indicated by bars with thick stripes) = 0 μM α-MSH + 0 μM agouti (**A,B**), 0 μM agouti (**C,D**) and 0 μM α-MSH + 0 μM H89 (**E,F**). Error bars represent standard deviation. Statistical significance is defined as $p < 0.05$ as compared to vehicle (indicated by *), or to 20 μM α-MSH + 0 μM agouti (**A**, indicated by #) and 20 μM α-MSH + 0 μM H89 (**F**, indicated by #), bars with thin stripes (**A-B** and **E-F**). The non-parametric Kruskal-Wallis test and the Mann-Whitney test were used for statistical analyses. α-MSH = α-melanocyte-stimulating hormone; H89 = protein kinase A (PKA) inhibitor; IL-6 = interleukin-6; MTT = 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide.

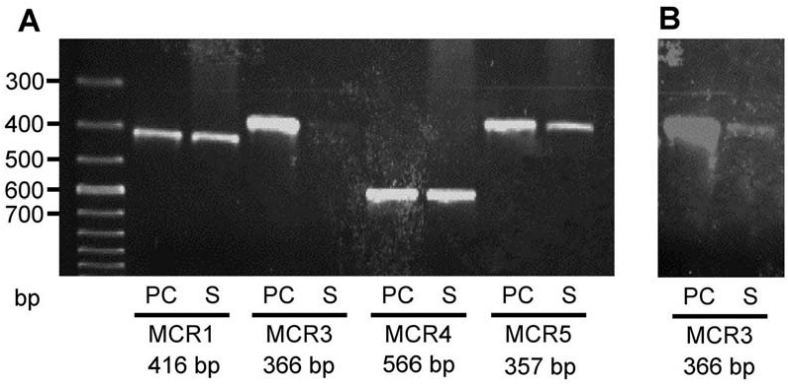

FIGURE 4 A-B RT-PCR analysis of total RNA extracted from cells of the human microglial cell line CHME3 using primers specific for the melanocortin receptor (MCR) subtypes MCR1, MCR3, MCR4 and MCR5, respectively. (**A**) The size of the bands is indicated by the number of base pairs (bp). Lane 1 = base pair ladder; lanes 2, 4, 6 and 8 = PCR-product from genomic DNA for MCR1, 3, 4 and 5, respectively; lanes 3, 5, 7 and 9 = RT-PCR-product from extracted RNA-samples for MCR1, 3, 4 and 5, respectively. (**B**) The figure demonstrates the same experiment after longer exposure to visualise the weak band for MCR3 mRNA. PC = positive control, *i.e.*, genomic DNA extracted from the CHME3 microglial cells; S = sample of RNA extracted from the CHME3 microglial cells.

increase in IL-6 release, and 30-40% reduction in cell viability (FIGs. 1 and 2). This dose of Aβ (10 μM) was used in the co-stimulatory experiments, since higher doses of $A\beta_{1-40}$ resulted in considerably larger reduction in cell viability.

Co-stimulation of the microglia with $A\beta_{1-40}$ (10 μM) and IFN-γ (1-100 ng/ml) did not result in a further increase in the IL-6 secretion, as compared to incubation with either $A\beta_{1-40}$ or IFN-γ alone (see FIG. 1E).

α-MSH, at 0.001-100 μM concentration, did not affect the secretion of IL-6 from the human microglial cells in response to Aβ or Aβ + IFN-γ (see FIG. 2C and 2E, respectively), whereas a small reduction in the basal release of IL-6 was observed at low concentrations (0.01 and 0.1 μM) of α-MSH (FIG. 2A). Higher concentrations of α-MSH (1 and 20 μM) resulted in a marked increase in the basal IL-6 secretion (FIG. 2A). Incubation with agouti resulted in a dose-dependent increase in the secretion of IL-6 induced by 20 μM α-MSH (FIG. 3A), as well as in the basal secretion of IL-6 (FIG. 3C). The PKA-inhibitor H89 (0.1-10 μM) had no effect on IL-6 release induced by 20 μM α-MSH (FIG. 3E).

Cell Viability

The time-response curve for $A\beta_{1-40}$ showed a marked decrease in cell viability of the human microglia after 24 h incubation at the concentration of 20 and 40 μM $A\beta_{1-40}$, and was further decreased at 48 h (data not shown). Freshly dissolved $A\beta_{1-40}$ (5-60 μM) resulted in a decreased viability of the human microglial cells in a dose-dependent manner (FIG. 1B). Incubation with IFN-γ alone (1-100 ng/ml) did not affect the cell viability (FIG. 1D), neither did IFN-γ affect the reduction in cell viability induced by 10 μM $A\beta_{1-40}$ (FIG. 1F).

α-MSH at concentrations 0.001-100 μM did not affect basal cell viability (FIG. 2B), nor the reduced cell viability observed after incubation Aβ (FIG. 2D) or Aβ + IFN-γ (FIG. 2F). Co-incubation with agouti (0.1-20 μM) and α-MSH (20 μM) induced a small, although not significant, reduction in the cell viability (FIG. 3B). Incubation with α-MSH (20 μM) and H89 resulted in a small dose-dependent decrease in the cell viability, as compared to vehicle or incubation with 20 μM α-MSH alone (FIG. 3F).

Expression of MCR Subtypes

RT-PCR analysis of RNA extracted from cells of the human microglial CHME3 cell line showed expression of MCR1, MCR3, MCR4 and MCR5 mRNA (FIG. 4). MCR4 mRNA was constitutively expressed in all of the 6 different passages analysed, whereas mRNA for MCR1 (4 of 6 experiments), MCR3 (1 of 6) and MCR5 (3 of 6) had varying expression. The size of the PCR products for each MCR subtype corresponded to the expected size, *i.e.*, 416 bp for MCR1, 366 bp for MCR3, 566 bp for MCR4, and 357 bp for MCR5, and to the size of the positive controls consisting of genomic DNA from the same human microglial cell line (FIG. 4). No signal was observed in the negative controls treated with DNase (not shown).

DISCUSSION

The presence of activated microglia within and around senile plaques suggests that Aβ, the major component in plaques, is involved in the activation of microglial cells in AD. *In vitro* studies provide evidence that Aβ peptides induce cytokine synthesis and release from microglial cells (Araujo and Cotman, 1992; Meda *et al.*, 1995; Murphy *et al.*, 1998; Combs *et al.*, 2001; Szczepanik *et al.*, 2001; Lee *et al.*, 2002; Takata *et al.*, 2003; Lindberg *et al.*, 2005). In the present study, we have investigated $A\beta_{1\text{-}40}$-induced activation of the human CHME3 microglial cell line and the effects thereupon of α-MSH, a neuropeptide with anti-inflammatory properties (see Catania and Lipton, 1998), with regard to cytokine production and cell viability. Freshly dissolved $A\beta_{1\text{-}40}$ stimulated the release of IL-6 from these cells in a dose-dependent manner, in accordance with studies using pre-aggregated $A\beta_{1\text{-}42}$ on human primary microglia (Lue *et al.*, 2001).

α-MSH did not affect the release of IL-6 from human microglial cells stimulated with $A\beta_{1\text{-}40}$, or with $A\beta_{1\text{-}40}$ and IFN-γ. In studies on the aggregated form of $A\beta_{1\text{-}42}$, the major form in amyloid plaques in the AD brain, α-MSH had an inhibitory effect on the release of TNF-α and NO (Galimberti *et al.*, 1999). Furthermore, α-MSH reduced the release of IL-6, TNF-α and NO, from murine microglia activated with LPS and IFN-γ (Delgado *et al.*, 1998). A possible explanation for the different effects of α-MSH is a species difference. To our knowledge, studies on the effects of α-MSH on microglia have, so far, been performed exclusively on murine cells (Delgado *et al.*, 1998; Galimberti *et al.*, 1999). Furthermore, the effects of α-MSH on Aβ-induced cytokine release may depend on the Aβ-form used. Indeed, previous studies on the effects of Aβ-peptides on microglia from different species have shown differential effects on cytokine release, depending on the aggregational form, and on the use of either $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ (Araujo and Cotman, 1992; Meda *et al.*, 1995; Murphy *et al.*, 1998; Combs *et al.*, 2001; Szczepanik *et al.*, 2001; Lee *et al.*, 2002; Takata *et al.*, 2003; Lindberg *et al.*, 2005). Freshly dissolved $A\beta_{1\text{-}40}$ was chosen for the present study since freshly dissolved Aβ-peptide was more potent than the fibrillar form and a further aggregated form, so called βamy balls (Westlind-Danielsson and Arnerup, 2001), in stimulating the release of pro-inflammatory cytokines from primary rat microglial cultures (Lindberg *et al.*, 2005). Aβ peptides are highly aggregational-prone, $A\beta_{1\text{-}42}$ more so than $A\beta_{1\text{-}40}$ (Jarrett *et al.*, 1993). The preparation of freshly dissolved $A\beta_{1\text{-}40}$ used in the present study was shown previously to contain mainly oligomeric forms, such as tetramers, hexamers, dodecamers and 24-mers, while the preparation of freshly dissolved $A\beta_{1\text{-}42}$ was shown to contain a mixture of oligomers and fibrils during 24h of incubation in culture medium (Lindberg *et al.*, 2005). Based on the findings that the oligomeric forms, *i.e.*, so called Aβ-derived diffusible ligands (ADDLs), believed to represent the most neurotoxic forms (Aksenova *et al.*, 1996; Hartley *et al.*, 1999; Dahlgren *et al.*, 2002; Selkoe, 2002; Hoshi *et al.*, 2003), $A\beta_{1\text{-}40}$, and not $A\beta_{1\text{-}42}$, was chosen for the present study. The human microglial cell line responded to the preparation of freshly dissolved $A\beta_{1\text{-}40}$ with a markedly reduced cell viability. This reduction in cell viability was not affected by IFN-γ or α-MSH. Interestingly, inhibition of PKA with H89, together with α-MSH, gave rise to a decrease in cell viability. PKA, a downstream mediator of MCR signalling, has been shown to activate murine microglial cells not previously activated (Min *et al.*, 2004). However, inhibition of PKA did not reduce the α-MSH-induced release of IL-6 from the human microglia. These findings indicate that PKA-signalling is involved in the reduction of cell viability, but not in mediating the release of IL-6. Although increased cAMP-signalling upon α-MSH stimulation inhibits the activation of murine microglia (Konda *et al.*, 1994; Delgado *et al.*, 1998), increased cAMP may have both stimulatory (Pyo *et al.*, 1999; Marcus *et al.*, 2003; Woo *et al.*, 2004) and inhibitory (Yoshimura *et al.*, 1997; Si *et al.*, 1998) effects on the release of inflammatory factors.

Monocytes have previously been shown to express the MCR1, 3 and 5 subtypes (Rajora *et al.*, 1996; Ichiyama *et al.*, 2000), and we now show the expression of mRNA for these receptors, as well as MCR4, in the human microglial cell line, suggesting that all of these receptors are possible candidates for mediating the effects of α-MSH on these cells. However, the endogenous antagonist of α-MSH-mediated activity, agouti, specific for MCR1 and MCR4 (Dinulescu and Cone, 2000), did not inhibit the α-MSH-induced release of IL-6. Instead, agouti further increased the α-MSH-induced release of IL-6. These findings would seem to indicate that α-MSH-induced secretion of IL-6 was mediated by MCR3 and/or MCR5. Agouti has some affinity for both of these MCRs (Dinulescu and Cone, 2000), but further studies will be necessary to establish whether it is sufficient to affect the α-MSH-induced stimulation of the human microglia.

There is ample evidence for anti-inflammatory effects of α-MSH (see Catania and Lipton, 1998), and the present finding that α-MSH stimulates, rather than inhibits the release of IL-6, is intriguing. Many studies show neuroprotective and regenerative activities of α-MSH (see Gispen *et al.*, 1994; Strand, 1999; Catania

et al., 2004) as well as of IL-6 (Maeda *et al.*, 1994; Loddick *et al.*, 1998; Streit *et al.*, 2000; Poulsen *et al.*, 2005) in the nervous system. Recent studies have demonstrated neuroprotective effects of α-MSH in models of cerebral ischemia (Huh *et al.*, 1997; Hwang *et al.*, 2004; Forslin Aronsson *et al.*, submitted). Thus, it may be hypothesised that α-MSH, through the release of IL-6 from microglial cells, can have beneficial effects on neurons.

The microglial cell line, CHME3, used in the present studies has previously been reported to express receptors for IFN-γ and to respond with increased production of major histocompatibility (MHC) class II molecules upon stimulation with IFN-γ (Janabi *et al.*, 1995). The activation of these cells by IFN-γ was supported by our results showing a dose-dependent increase in the release of IL-6. However, unlike earlier studies on murine microglia (Gasic-Milenkovic *et al.*, 2003), there was no potentiating effect of IFN-γ on the $A\beta_{1\text{-}40}$-induced secretion of IL-6. This could be due to the fact that IFN-γ and IL-6 share the same signalling pathway, *i.e.*, the JAK/STAT pathway (Heinrich *et al.*, 1998; Platanias and Fish, 1999; Kerr *et al.*, 2003). Previous characterisation of the CHME3 cells has also demonstrated that they have the same repertoire of chemokine receptors as primary cultures of adult human microglia (Flynn *et al.*, 2003), and that their responses to chemokines are similar to those of primary cultures of adult rat microglia (Cross and Woodroofe, 1999). This is interesting in view of the findings of increased chemokine levels in CSF samples from AD patients (Galimberti *et al.*, 2003), and supports the relevance of using this cell line as a model for human microglia.

The results from the present study support the hypothesis that $A\beta_{1\text{-}40}$ activates microglial cells to produce IL-6 in the AD brain, which could further stimulate the production of $A\beta_{1\text{-}40}$, via stimulation of APP synthesis and processing (Del Bo *et al.*, 1995). This would promote chronic inflammation that is believed to be part of AD brain pathology. Unexpectedly, α-MSH was not found to inhibit the release of IL-6, but resulted in increased basal secretion from the microglial cells. Further studies will be required to clarify whether or not this effect is responsible for the neuroprotective activities of α-MSH.

Acknowledgements

This work was supported by grants from the Swedish Research Council (072194), Stiftelsen för Gamla Tjänarinnor, Gun och Bertil Stohnes Stiftelse, Insamlingsstiftelsen för Alzheimer-och Demensforskning, Stiftelsen för åldersforskning and Stiftelsen Demensfonden.

References

Adan RA and WH Gispen (1997) Brain melanocortin receptors: from cloning to function. *Peptides* **18**, 1279-1287.

Aksenova MV, MY Aksenov, DA Butterfield and JM Carney (1996) α-1-antichymotrypsin interaction with Aβ (1-40) inhibits fibril formation but does not affect the peptide toxicity. *Neurosci. Lett.* **211**, 45-48.

Araujo DM and CW Cotman (1992) β-amyloid stimulates glial cells *in vitro* to produce growth factors that accumulate in senile plaques in Alzheimer's disease. *Brain Res.* **569**, 141-145.

Bauer J, S Strauss, U Schreiter-Gasser, U Ganter, P Schlegel, I Witt, B Yolk and M Berger (1991) Interleukin-6 and α-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices. *FEBS Lett.* **285**, 111-114.

Benveniste EN, VT Nguyen and GM O'Keefe (2001) Immunological aspects of microglia: relevance to Alzheimer's disease. *Neurochem. Int.* **39**, 381-391.

Blum-Degen D, T Muller, W Kuhn, M Gerlach, H Przuntek and P Riederer (1995) Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and *de novo* Parkinson's disease patients. *Neurosci. Lett.* **202**, 17-20.

Böhm M, M Schiller, S Stander, H Seltmann, Z Li, T Brzoska, D Metze, HB Schiöth, A Skottner, K Seiffert, CC Zouboulis and TA Luger (2002) Evidence for expression of melanocortin-1 receptor in human sebocytes *in vitro* and *in situ*. *J. Invert. Dermatol.* **118**, 533-539.

Cacabelos R, M Barquero, P Garcia, XA Alvarez and E Varela de Seijas (1991) Cerebrospinal fluid interleukin-1 beta (IL-1β) in Alzheimer's disease and neurological disorders. *Methods Fund. Exp. Clin. Pharmacol.* **13**, 455-458.

Cammas FM, S Kapas, S Barker and AJ Clark (1995) Cloning, characterization and expression of a functional mouse ACTH receptor. *Biochem. Biophys. Res. Commun.* **212**, 912-918.

Catania A and JM Lipton (1998) Peptide modulation of fever and inflammation within the brain. *Ann. NY Acad. Sci.* **856**, 62-68.

Catania A, S Gatti, G Colombo and JM Lipton (2004) Targeting melanocortin receptors as a novel strategy to control inflammation. *Pharmacol. Rev.* **56**, 1-29.

Combs CK, JC Karlo, SC Kao and GE Landreth (2001) β-Amyloid stimulation of microglia and monocytes results in TNFα-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J. Neurosci.* **21**, 1179-1188.

Cone RD (2005) Anatomy and regulation of the central melanocortin system. *Nat. Neurosci.* **8**, 571-578.

Cross AK and MN Woodroofe (1999) Chemokines induce migration and changes in actin polymerization in adult rat brain microglia and human fetal microglial cell line *in vitro*. *J. Neurosci. Res.* **55**, 17-23.

Dahlgren KN, AM Manelli, WB Stine Jr, LK Baker, GA Krafft and MJ LaDu (2002) Oligomeric and fibrillar species of amyloid-peptides differentially affect neuronal viability. *J. Biol. Chem.* **277**, 32046-32053.

Del Bo R, N Angeretti, E Lucca, MG De Simoni and G Forloni (1995) Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and β-amyloid production in cultures. *Neurosci. Lett.* **188**, 70-74.

Delgado R, A Carlin, L Airaghi, MT Demitri, L Meda, D Galimberti, P Baron, JM Lipton and A Catania (1998) Melanocortin peptides

inhibit production of proinflammatory cytokines and nitric oxide by activated microglia. *J. Leukoc. Biol.* **63**, 740-745.

Dickson DW, SC Lee, LA Mattiace, SH Yen and C Brosnan (1993) Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia* **7**, 75-83.

Dinulescu DM and RD Cone (2000) Agouti and agouti-related protein: analogies and contrasts. *J. Biol. Chem.* **275**, 6695-6698.

Eikelenboom P, SS Zhan, WA van Gool and D Allsop (1994) Inflammatory mechanisms in Alzheimer's disease. *Trends Pharmacol. Sci.* **15**, 447-450.

Flynn G, S Maru, J Loughlin, IA Romero and D Male (2003) Regulation of the chemokine receptor expression in human microglia and aastrocytes. *J. Neuroimmunol.* **136**, 84-93.

Galimberti D, P Baron, L Meda, E Prat, E Scarpini, R Delgado, A Catania, JM Lipton and G Scarlato (1999) α-MSH peptides inhibit production of nitric oxide and tumor necrosis factor-α by microglial cells activated with β-amyloid and interferon γ. *Biochem. Biophys. Res. Commun.* **263**, 251-256.

Galimberti D, N Schoonenboom, E Scarpini and P Scheltens (2003) Chemokines in serum and cerebrospinal fluid of Alzheimer's disease patients. **Ann. Neurol.** *53*, 547-548.

Gasic-Milenkovic J, S Dukic-Stefanovic, W Deuther-Conrad, U Gärtner and G Münch (2003) β-amyloid peptide potentiates inflammatory responses induced by lipopolysaccharide, interferon-γ and 'advanced glycation endproducts' in a murine microglia cell line. *Eur. J. Neurosci.* **17**, 813-821.

Gispen WH, J Verhaagen and D Bar (1994) ACTH/MSH-derived peptides and peripheral nerve plasticity: neuropathies, neuroprotection and repair. *Prog. Brain Res.* **100**, 223-229.

Giulian D, LJ Haverkamp, JH Yu, W Karshin, D Tom, J Li, J Kirkpatrick, LM Kuo and AE Roher (1996) Specific domains of β-amyloid from Alzheimer plaque elicit neuron killing in human microglia. *J. Neurosci.* **16**, 6021-6037.

Glyn JR and JM Lipton (1981) Hypothermic and antipyretic effects of centrally administered ACTH (1-24) and α-melanotropin. *Peptides* **2**, 177-187.

Griffin WS, LC Stanley, C Ling, L White, V MacLeod, LJ Perrot, CL White 3rd and C Araoz (1989) Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **86**, 7611-7615.

Hampel H, D Schoen, MJ Schwarz, HU Kotter, C Schneider, T Sunderland, R Dukoff, J Levy, F Padberg, S Stubner, K Buch, N Muller and HJ Moller (1997) Interleukin-6 is not altered in cerebrospinal fluid of first-degree relatives and patients with Alzheimer's disease. *Neurosci. Lett.* **228**, 143-146.

Hartley DM, DM Walsh, CP Ye, T Diehl, S Vasquez, PM Vassilev, DB Teplow and DJ Selkoe (1999) Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* **19**, 8876-8884.

Heinrich PC, I Behrmann, G Muller-Newen, F Schaper and L Graeve (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**, 297-314.

Hoshi M, M Sato, S Matsumoto, A Noguchi, K Yasutake, N Yoshida and K Sato (2003) Spherical aggregates of β-amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3β. *Proc. Natl. Acad. Sci. USA* **100**, 6370-6375.

Huh SK, JM Lipton and HH Batjer (1997) The protective effects of α-melanocyte stimulating hormone on canine brain stem ischemia. *Neurosurgery* **40**, 132-139.

Hwang IK, KY Yoo, JK Park, YS Nam, IS Lee, JH Kang, SY Choi, JY Lee, TC Kang and MH Won (2004) Ischemia-related changes of adrenocorticotropic hormone immunoreactivity and its protective effect in the gerbil hippocampus after transient forebrain ischemia. *Neuroscience* **126**, 871-877.

Ichiyama T, S Sato, K Okada, A Catania and JM Lipton (2000) The neuroimmunomodulatory peptide α-MSH. *Ann. NY Acad. Sci.* **917**, 221-226.

Janabi N, S Peudenier, B Heron, KH Ng and M Tardieu (1995) Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci. Lett.* **195**, 105-108.

Jarrett JT, EP Berger and PT Lansbury Jr (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693-4697.

Kerr IM, AP Costa-Pereira, BF Lillemeier and B Strobl (2003) Of JAKs, STATs, blind watchmakers, jeeps and trains. *FEBS Lett.* **546**, 1-5.

Konda Y, I Gantz, J DelValle, Y Shimoto, H Miwa and T Yamada (1994) Interaction of dual intracellular signaling pathways activated by the melanocortin-3 receptor. *J. Biol. Chem.* **269**, 13162-13166.

Lee YB, A Nagai and SU Kim (2002) Cytokines, chemokines, and cytokine receptors in human microglia. *J. Neurosci. Res.* **69**, 94-103.

Lindberg C, M-L Bardyl Selenica, A Westlind-Danielsson and M Schultzberg (2005) β-amyloid structure determines the nature of cytokine release from rat microglia. *J. Mol. Neurosci.* **27**, 1-12.

Loddick SA, AV Turnbull and NJ Rothwell (1998) Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.* **18**, 176-179.

Lue LF, R Rydel, EF Brigham, LB Yang, H Hampel, GM Murphy Jr, L Brachova, SD Yan, DG Walker, Y Shen and J Rogers (2001) Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia *in vitro*. *Glia* **35**, 72-79.

Maeda Y, M Matsumoto, O Hori, K Kuwabara, S Ogawa, SD Yan, T Ohtsuki, T Kinoshita, T Kamada and DM Stern (1994) Hypoxia/reoxygenation-mediated induction of astrocyte interleukin 6: a paracrine mechanism potentially enhancing neuron survival. *J. Exp. Med.* **180**, 2297-2308.

Marcus JS, SL Karackattu, MA Fleegal and C Sumners (2003) Cytokine-stimulated inducible nitric oxide synthase expression in astroglia: role of Erk mitogen-activated protein kinase and NF-κB. *Glia* **41**, 152-160.

Martinez M, E Fernandez-Vivancos, A Frank, M De la Fuente and A Hernanz (2000) Increased cerebrospinal fluid fas (Apo-1) levels in Alzheimer's disease. Relationship with IL-6 concentrations. *Brain Res.* **869**, 216-219.

McGeer EG and PL McGeer (2003) Inflammatory processes in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **27**, 741-749.

Meda L, MA Cassatella, GI Szendrei, L Otvos Jr, P Baron, M Villalba, D Ferrari and F Rossi (1995) Activation of microglial cells by β-amyloid protein and interferon-γ. *Nature* **374**, 647-650.

Min KJ, MS Yang, I Jou and EH Joe (2004) Protein kinase A mediates microglial activation induced by plasminogen and gangliosides. *Exp. Mol. Med.* **36**, 461-467.

Mountjoy KG, LS Robbins, MT Mortrud and RD Cone (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248-1251.

Murphy GM Jr, L Yang and B Cordell (1998) Macrophage colony-stimulating factor augments β-amyloid-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. *J.*

Biol. Chem. **273**, 20967-20971.

Neuroinflammation working group, H Akiyama, S Barger, S Barnum, B Bradt, J Bauer, GM Cole, NR Cooper, P Eikelenboom, M Emmerling, BL Fiebich, CE Finch, S Frautschy, WS Griffin, H Hampel, M Hull, G Landreth, L Lue, R Mrak, IR Mackenzie, PL McGeer, MK O'Banion, J Pachter, G Pasinetti, C Plata-Salaman, J Rogers, R Rydel, Y Shen, W Streit, R Strohmeyer, I Tooyoma, FL Van Muiswinkel, R Veerhuis, D Walker, S Webster, B Wegrzyniak, G Wenk and T Wyss-Coray (2000) Inflammation and Alzheimer's disease. *Neurobiol. Aging* **21**, 383-421.

Oka T, K Oka, M Hosoi and T Hori (1996) Inhibition of peripheral interleukin-1β-induced hyperalgesia by the intracerebroventricular administration of diclofenac and α-melanocyte-stimulating hormone. *Brain Res.* **736**, 237-242.

Platanias LC and EN Fish (1999) Signaling pathways activated by interferons. *Exp. Hematol.* **27**, 1583-1592.

Poulsen CB, M Penkowa, R Borup, FC Nielsen, M Caceres, A Quintana, A Molinero, J Carrasco, M Giralt and J Hidalgo (2005) Brain response to traumatic brain injury in wild-type and interleukin-6 knockout mice: a microarray analysis. *J. Neurochem.* **92**, 417-432.

Pyo H, I Jou, S Jung and E Joe (1999) cAMP potentiates β-amyloid-induced nitric oxide release from microglia. *Neuroreport* **10**, 37-40.

Rajora N, G Ceriani, A Catania, RA Star, MT Murphy and JM Lipton (1996) alpha-MSH production, receptors, and influence on neopterin in a human monocyte/macrophage cell line. *J. Leukoc. Biol.* **59**, 248-253.

Selkoe DJ (1994) Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease. *Annu. Rev. Cell Biol.* **10**, 373-403.

Selkoe DJ (2002) Deciphering the genesis and fate of amyloid β-protein yields novel therapies for Alzheimer disease. *J. Clin. Invest.* **110**, 1375-1381.

Si Q, Y Nakamura, T Ogata, K Kataoka and P Schubert (1998) Differential regulation of microglial activation by propentofylline via cAMP signaling. *Brain Res.* **812**, 97-104.

Strand FL (1999) New vistas for melanocortins. Finally, an explanation for their pleiotropic functions. *Ann. NY Acad. Sci.* **897**, 1-16.

Streit WJ, SD Hurley, TS McGraw and SL Semple-Rowland (2000) Comparative evaluation of cytokine profiles and reactive gliosis supports a critical role for interleukin-6 in neuron-glia signaling during regeneration. *J. Neurosci. Res.* **61**, 10-20.

Szczepanik AM, D Rampe and GE Ringheim (2001) Amyloid-β peptide fragments p3 and p4 induce pro-inflammatory cytokine and chemokine production *in vitro* and *in vivo*. *J. Neurochem.* **77**, 304-317.

Takata K, Y Kitamura, M Umeki, D Tsuchiya, J Kakimura, T Taniguchi, PJ Gebicke-Haerter and S Shimohama S (2003) Possible involvement of small oligomers of amyloid-β peptides in 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2-sensitive microglial activation. *J. Pharmacol. Sci.* **91**, 330-333.

Tarkowski E, K Blennow, A Wallin and A Tarkowski (1999) Intracerebral production of tumor necrosis factor-α, a local neuroprotective agent, in Alzheimer disease and vascular dementia. *J. Clin. Immunol.* **19**, 223-230.

Tatro JB (1996) Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. *Neuroimmunomodulation* **3**, 259-284.

Vandenabeele P and W Fiers (1991) Is amyloidogenesis during Alzheimer's disease due to an IL-1-/IL-6-mediated 'acute phase response' in the brain? *Immunol. Today* **12**, 217-219.

Vehmas AK, CH Kawas, WF Stewart and JC Troncoso (2003) Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. *Neurobiol. Aging* **24**, 321-331.

Westlind-Danielsson A and G Arnerup (2001) Spontaneous *in vitro* formation of supramolecular β-amyloid structures, "βamy balls", by β-amyloid 1-40 peptide. *Biochemistry* **40**, 14736-14743.

Wikberg JE (1999) Melanocortin receptors: perspectives for novel drugs. *Eur. J. Pharmacol.* **375**, 295-310.

Woo MS, SH Jung, JW Hyun and HS Kim (2004) Differential regulation of inducible nitric oxide synthase and cytokine gene expression by forskolin and dibutyryl-cAMP in lipopolysaccharide-stimulated murine BV2 microglial cells. *Neurosci. Lett.* **356**, 187-190.

Yoshimura T, C Kurita, T Nagao, E Usami, T Nakao, S Watanabe, J Kobayashi, F Yamazaki, H Tanaka, N Inagaki and H Nagai (1997) Inhibition of tumor necrosis factor-α and interleukin-1-β production by β-adrenoceptor agonists from lipopolysaccharide-stimulated human peripheral blood mononuclear cells. *Pharmacology* **54**, 144-152.

II

Effects of immunomodulatory substances on phagocytosis of $A\beta_{1-42}$ by human microglia

Erik Hjorth [a,] *, Dan Frenkel [b], Howard Weiner [c], and Marianne Schultzberg [a]

[a] Department of Neurobiology, Care Sciences and Society, Division of Neurodegeneration, Karolinska Institutet, SE-141 86 Stockholm, Sweden

[b] Department of Neurobiology, Tel Aviv University, Tel Aviv, 69978, Israel

[c] Center for Neurologic Disease, Harvard Medical School, Brigham & Womens Hospital, 75 Francis Street, Boston, MA 02115 USA

* Correspondence to:
Marianne Schultzberg
Karolinska Institutet
Dept. of Neurobiology, Care Sciences and Society
Division of Neurodegeneration
Novum, 5th floor
SE-141 86 Stockholm
Sweden
Tel: +46-8-585 83881
Fax: +46-8-585 83880
Email: marianne.schultzberg@ki.se

Abstract

Glial activation and increased inflammation characterize neuropathology in Alzheimer's disease (AD). The aim was to develop a model for studying phagocytosis of β-amyloid (Aβ) peptide by human microglia, and to test effects thereupon by immunomodulatory substances. Human CHME3 microglia showed intracellular $A\beta_{1-42}$ co-localized with lysosome-associated membrane protein-2, indicating phagocytosis. This was increased by interferon-γ, and to a lesser degree with Protollin, a proteosome-based adjuvant. Secretion of brain-derived neurotrophic factor (BDNF) was decreased by $A\beta_{1-42}$ and by interferon-γ and interleukin-1β. These cytokines, but not $A\beta_{1-42}$, stimulated interleukin-6 release. Microglia which phagocytosed $A\beta_{1-42}$ exhibited a higher degree of expression of interleukin-1 receptor type I and inducible nitric oxide synthase. In conclusion, we show that human microglia are able to phagocytose $A\beta_{1-42}$ and that this is associated with expression of inflammatory markers. $A\beta_{1-42}$ and interferon-γ decrease BDNF secretion suggesting a new neuropathological role for $A\beta_{1-42}$ and the inflammation accompanying AD.

Key words: Alzheimer, amyloid, BDNF, brain-derived neurotrophic factor, cytokine, interferon, interleukin, Protollin

Introduction

Alzheimer's disease (AD) is the most common cause of dementia. The major pathological hallmarks of AD besides neuronal loss are amyloid plaques and neurofibrillary tangles (NFTs). Both amyloid plaques and NFTs have been implicated in neuronal impairment and death in a large number of studies. Although a great deal of controversy exists about the relative importance of amyloid plaques *contra* NFT:s in AD [1], there is an overwhelming body of evidence showing that amyloid plaques and the peptides they are composed of are culprits in the neurodegenerative processes in AD [2]. The main component of amyloid plaques is the β-amyloid (Aβ) peptide, that is secreted by neurons and other cells through cleavage of the larger, membrane-bound, amyloid precursor protein (APP). APP can be processed by two major pathways: the amyloidogenic pathway that yields Aβ, and the non-amyloidogenic yielding fragments believed to be non-pathogenic. The Aβ peptides are prone to self-aggregation and deposition into insoluble plaques. This is especially true for the 42 amino acid form ($A\beta_{1\text{-}42}$), which is the predominant form in the dense core plaques [3]. Aβ-species also exist in the forms of soluble monomers and oligomers which similarly to the amyloid plaques are more abundant in the AD brain than in the non-AD brain [4]. Somehow, the balance between production and clearance/degradation of Aβ is disturbed in AD.

Damage to brain tissue induces an inflammation, a response which is present in many, if not all neurodegenerative conditions. In the central nervous system (CNS), glial cells, *i.e.* microglia and astrocytes, represent the main source of inflammatory reactions. In normal conditions, glia have supportive functions, including maintenance of ionic homeostasis, clearance of neurotransmitters and in the case of astrocytes, providing nutrients to the energy-demanding work of the neurons [5]. Glial, particularly microglial, cell responses can also serve in the elimination of debris from damaged cells and to remove pathogens. Removal of pathogens is executed by the process of phagocytosis, a capability that glial cells share with peripheral immunocompetent cells including monocytes and macrophages. In the brain, phagocytosis is believed to be performed mainly by microglia, but astrocytes also have this capability [6].

Under stress, glial cells proliferate and become activated, which leads to production of neurotoxic molecules such as free radical species and proinflammatory cytokines [7,8]. In contrast to the potentially tissue-damaging responses, glia can also produce factors that promote neuroprotection and neuronal plasticity [9]. These protective and supportive

functions may be downregulated during stress. Therefore, inflammation in the brain may worsen the outcome of an already existing trauma or pathological condition.
In the AD brain, activated microglia and astrocytes are present in the areas of neurodegeneration and amyloid plaques [10] and there is an increased production of proinflammatory cytokines such as interleukin (IL) -1 [11] and IL-6 [12]. In addition, elevated levels of IL-1 and IL-6 have been found in serum and cerebrospinal fluid (CSF) from AD patients [13,14]. *In vitro* studies have demonstrated that Aβ peptides can indeed activate glial cells to produce inflammatory factors [15-18], which can contribute to the neurodegenerative process [19]. Evidence from studies on rat cortical microglia showed that the smaller aggregational forms of Aβ such as oligomers are more potent in stimulating glial secretion of proinflammatory cytokines [18]. IL-1 increases the production of APP in human [20] glia, and the APP gene contains a binding site for the prime inflammatory transcription factor nuclear factor κB (NFκB) [21]. Furthermore, inflammation may shift processing of APP towards the amyloidogenic pathway [22]. A consequence of the interactions between inflammation and the APP/Aβ-peptide may be a vicious circle [23], in which inflammation increases Aβ levels through increased production and reduced clearance, which in turn results in neuronal cell death and a perpetuated and increased glial activation and release of proinflammatory factors, as well as neuronal cell death, and so forth.

Removal of a disease-causing pathogen is probably the most effective way of treating a disease. To stimulate cellular uptake, phagocytosis, of Aβ is a promising strategy. In studies on animal models of AD, active and passive immunizations have been shown to be effective in removing plaques and to improve cognitive performance [24]. Human clinical trials with active immunization have been started, but were aborted due to serious sideeffects in a few cases [25]. However, several clinical trials with modified protocols, including passive immunizations, are currently being carried out. Phagocytosis is an activity that is performed primarily by cells of the immune system, notably cells of the monocyte lineage. In the light of the studies on active and passive immunization we are presented with a strategy for treatment of AD, to activate the immune system into phagocytosis of Aβ. Although inflammation generally is believed to stimulate phagocytic activities, there is also evidence indicating that inflammation may inhibit phagocytosis [26], whereas anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) can stimulate phagocytosis [27]. Thus, it is of importance to search for compounds that can promote phagocytosis without starting the

damaging processes of inflammation. In short, directed and differential activation of immune cells residing in, or destined for, the CNS constitutes a potential therapeutic target.

In the present study, the aim was to characterise the responses of human microglia to the exposure of Aβ. In order to investigate the possibilities to increase glial uptake of Aβ we have analysed the effects of different immunomodulatory substances. The effects of the adjuvant Protollin, as well as those of the archetypical proinflammatory cytokines, IL-1β and interferon-γ (IFNγ), were analysed in an *in vitro* model of human microglial cells with regard to $A\beta_{1\text{-}42}$ uptake, microglial phenotype, and the secretion of IL-6 and brain-derived neurotrophic factor (BDNF).

IL-6 is a cytokine that is induced by IL-1β and tumour necrosis factor-α (TNFα) and can thus be considered a general measure of inflammation [28]. BDNF is a neurotrophic growth factor of importance in memory formation and neuroprotection [29,30]. Protollin is an adjuvant made of *Shigella flexneri* 2a lipopolysaccharides (LPS) associated non-covalently to meningococcal outer membrane proteins (proteosomes), that has been proven to be safe for use in humans [31]. While LPS activates Toll-like receptor type 4 (TLR4), cd11 and cd14, proteosomes activate TLR2 [32]. Activation of TLR2 has been associated with increased phagocytosis in mice with sciatic nerve lesions [33]. Furthermore, intranasal application of Protollin was shown to prevent accumulation of Aβ in young transgenic mice expressing the human APP with the Swedish mutation, and also to stimulate clearance of Aβ from the brain of aged mice of the same transgenic strain [34]. A correlation was found between the removal of Aβ and the level of microglial activation, as demonstrated by increased expression of the activation marker CD11b in conjunction with the removal of Aβ in animals treated with Protollin [34]. To investigate the effects of Protollin on cellular inflammatory markers, and the association of these markers with uptake of $A\beta_{1\text{-}42}$ by the human microglial cells, the expression of inducible nitric oxide synthase (iNOS), IL-1β and the signalling type I receptor for IL-1β (IL-1RI) was analyzed in cells showing uptake of $A\beta_{1\text{-}42}$. iNOS is induced in inflammation and shown to be harmful for neurons due to the production of radical nitrogen species [35]. It has been shown to be associated with neurodegenerative disorders such as AD [36].

Materials and Methods

Chemicals

Protollin was provided by Glaxo-Smith Kline Biologicals, Laval, Quebec, Canada. $A\beta_{1-42}$ conjugated with HiLyteFluor488 or biotin was obtained from Anaspec (Fremont, USA). Dimethylsulfoxide (DMSO), Triton-X100, bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma, Stockholm, Sweden. Normal donkey and goat serum, and fluorescence mounting medium (DakoPatts, Stockholm, Sweden). Streptavidin-7-amino-4-methyl-3-coumarinylacetic acid (AMCA) (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Lactate deydrogenase (LDH)-assay (Roche, Stockholm, Sweden). ELISA-kits for IL-6 and BDNF (R&D systems, Abingdon, United Kingdom). IFNγ (Bachem, Weil am Rhein, Germany). Cell culture medium, phosphate-buffered saline (PBS), GlutaMaxII, foetal calf serum (FCS) and PBS-based enzyme-free cell dissociation buffer (Invitrogen, Stockholm, Sweden). Cell culture bottles and multi-well plates (BD Biosciences, Stockholm, Sweden).

Cell cultures

Human microglial cells (CHME3) were obtained as a kind gift from Prof. M Tardieu (Neurologie pédiatrique, Hôpital Bicêtre, Assistance publique, Hôpitaux de Paris, Paris, France). CHME3 cells were cultured in T75 or T175 bottles in culture medium (DMEM/high glucose w/o sodium pyruvate supplemented with 2 mM L-glutamine or GlutaMaxII and 10% heat-inactivated FCS). The cells were subcultured at confluence using enzyme-free cell dissociation buffer after washing once with PBS without Mg^{2+} and Ca^{2+}.

Experimental procedures

The CHME3 microglial cells were seeded in 48-well plates for analysis of cell viability (MTT) and cytotoxicity (LDH) (see Supplementary material), in 6-well plates for flow-cytometry and analysis of substances released to the medium, or on glass coverslips in 24- or 48-well plates for immunocytochemistry. All experiments were performed at a confluence of ~60%. $A\beta_{1-42}$ was dissolved in DMSO and stored in darkness at $+4^{o}C$ until use at a final concentration of 1 μg/ml for all experiments except for a dose-response curve. In higher doses we observed aggregate-like precipitates of fluorescent $A\beta_{1-42}$ which were unwanted in this study. Before the addition of $A\beta_{1-42}$ or vehicle (DMSO), the cells were pre-stimulated for 24 h with either Protollin (0.001, 0.01 and 0.1 μg/ml in serum-free culture medium) or cytokines

(50 ng/ml IL-1β, 50 ng/ml IFNγ, or IL-1β + IFNγ). At 0, 24, 48, 72 and 96 h after addition of $A\beta_{1-42}$ the cultures were analyzed for uptake of $A\beta_{1-42}$, expression of cellular markers and secretory products. Uptake of fluorescent $A\beta_{1-42}$ by living cells was analysed in a Nikon TE600 inverted fluorescence microscope. Cell viability and cell death were also analysed (see Supplementary material).

Quantification of $A\beta_{1-42}$ phagocytosis and cellular markers by flow-cytometry

After the experimental treatment, the CHME3 microglial cells were dissociated with PBS-based enzyme-free dissociation buffer as described above, and centrifuged at 1500 x g for 10 min. The cells were then resuspended and fixed in 1% para-formaldehyde (PF) in PBS, for 40 min at room temperature, after which the fixative was diluted 20X by addition of PBS and the cells were centrifuged at 1500 x g for 10 min. Fixation with PF renders cells permeable to PI and therefore all fixed cells will be stained with PI, allowing the distinction of cells from cell debris. Analysis was performed in a FACScalibur (BD) flow-cytometer. A detected event was defined as a cell if it was gated through the front-scatter (FSC) and side-scatter (SSC) plot gate in addition to being positive for PI. A cell positive for phagocytosis of $A\beta_{1-42}$ ($A\beta_{1-42}$+) was defined as a PI-positive cell that was also being positive for the fluorophore conjugated to $A\beta_{1-42}$ (HyliteFluor488). Negative controls were utilized to establish the limits of detection for positive signals.

To investigate the phenotype of the cells with regard to the presence of inflammatory markers, and the degree of co-localization of each marker with phagocytosed $A\beta_{1-42}$, the cells were stained with antibodies directed against human IL-1β (1:400; gift from Dr. Stefan Svensson, Statens Bakteriologiska Laboratorium, Stockholm, Sweden), IL-1RI (1:200; Amgen (Immunex Corporation) Thousand Oaks, USA) and iNOS (1:600; R&D systems, London, England). The cells were harvested and fixed as described above and subsequently an aliquot of the cell suspension was incubated with the primary antibodies, diluted in PBS containing 5% normal donkey serum and 0.1% Triton-X100. Omission of primary antibodies served as negative control to establish the limits of detection for positive signals. After incubation with primary antibodies overnight at +4^{o}C, the cell suspension was washed by addition of PBS followed by centrifugation at 2500 x g for 20 min. The cells were resuspended in PBS and incubated with donkey anti-goat IgG-NL637 antibodies (1:500; R&D systems, London, England) and PI for 1 h at room temperature. After incubation, the cell suspension was diluted with PBS and analyzed by flow-cytometry. A cell displaying

immunoreactivity for an antibody was defined as a cell showing a stronger signal in this channel than the negative control. A cell displaying uptake of $A\beta_{1-42}$ ($A\beta_{1-42}$+) was defined as described above. The results were analysed from a scatter plot with fluorescence from the secondary antibody on the y-axis and fluorescence from HyliteFluor488 on the X-axis, divided into quadrants with borders based on the negative controls as described. The cells were thus being viewed in a two-way binary fashion with a cell being present in a certain quadrant thus indicating: a) positive for immunoreactivity to IL-1β (IL-1β+), IL-1RI (IL-1RI+) or iNOS (iNOS+) and for $A\beta_{1-42}$-uptake ($A\beta_{1-42}$+), b) positive for immunoreactivity to one of these markers and negative for $A\beta_{1-42}$-uptake c) negative for immunoreactivity to the markers and positive for $A\beta_{1-42}$-uptake, or d) negative for immunoreactivity to the markers and for $A\beta_{1-42}$-uptake. The parameters extracted from the data were a) total proportion of the cells showing immunoreactivity to a marker, b) immunoreactivity of cells negative for $A\beta_{1-42}$-uptake and c) immunoreactivity of cells positive for $A\beta_{1-42}$-uptake.

Immunocytochemistry and staining of fixed and living cells for microscopy

To analyse the microglial cell expression of certain inflammatory markers and the localization of phagocytosed $A\beta_{1-42}$ by microscopy the culture medium was removed and the coverslips dried at 37^{o}C for ~2 h. The cells were fixed with 4% PF for 20 min at room temperature, washed in PBS and incubated overnight at 4^{o}C with antibodies against IL-1β (1:400), IL-1RI (1:200) and iNOS (1:600), respectively. The antibodies were diluted in PBS containing 5% normal donkey serum and 0.1% Triton-X100. After rinsing in PBS, the coverslips were incubated for 1 h at room temperature with goat anti-rabbit-IgG conjugated with Cy2 (1:200), diluted in PBS containing 5% normal goat serum, 0.1% Triton-X100 and 1 µg/ml PI. For visualization of biotinylated $A\beta_{1-42}$, streptavidin-AMCA was included in the secondary antibody solution (2 µg/ml). The coverslips were then washed with PBS, mounted with fluorescence mounting medium, and inspected in a Nikon E800 microscope. To investigate the targeting of $A\beta_{1-42}$ toward degradation, cells were incubated with HyliteFluor488-conjugated $A\beta_{1-42}$ and then fixed and incubated overnight at 4^{o}C with mouse antibodies against human lysosome-associated membrane protein-2 (lamp-2, Millipore). After washing, the cells were incubated with donkey anti-mouse antibodies conjugated with Cy3 and with the nuclear stain DAPI, and then mounted and inspected as described above.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6 and BDNF in the cell culture medium were analyzed with commercially available ELISA-kits according to the manufacturer's instructions. Analysis of optical density (OD) was performed in a TECAN Safire2 plate reader.

Cell viability – MTT assay

To analyse cell viability the culture medium was removed and after washing with serum- and phenol-free DMEM/high glucose with GlutaMaxII, MTT (0.3 mg/ml) was added and the cells incubated at 37^{o}C for 1 h. The crystals formed by the reaction were dissolved in DMSO and the OD was measured with a TECAN Safire2 plate reader (Tecan, Mölndal, Stockholm) at 592 nm with 620 nm as a reference wavelength.

Cell death – LDH assay

To analyse cell death the culture medium was removed and added to a 96-well plate, and then incubated with LDH-reagent according to the manufacturer's instructions. The OD was measured at 492 nm with 620 nm as reference wavelength.

Statistics

The data were normalized to vehicle (MTT-assay, ELISA), $A\beta_{1-42}$ (flow-cytometry, for uptake) or positive control (LDH-assayu), or not normalized (flow-cytometry, for association of cellular markers with the uptake of $A\beta_{1-42}$, correlation between variables). The results were analysed with Kruskal-Wallis non-parametric analysis of variance (ANOVA) and the data were then compared pair-wise using the non-parametric Mann-Whitney U test with Bonferoni correction. Pair-wise comparison of variable within experimental groups was performed with the Wilcoxon Matched Pairs Test. Correlation between variables were performed with the Spearman Rank Order Correlations test. All statistical analyses were performed in Statistica v8 (Statsoft).

Results

Human CHME3 microglial cells exposed to immunomodulatory substances were studied with regard to phagocytosis of $A\beta_{1-42}$ and expression of cellular markers (IL-1β, IL-1RI and iNOS). As an indicator of an inflammatory response we analysed the secretion of IL-6 in the culture medium. To assess beneficial and neuroprotective activities the secretion of BDNF

was determined. The levels of BDNF released into the medium varied between 8 and 300 pg/ml and the levels of IL-6 varied between 20 and 950 pg/ml under basal conditions (vehicle group at 24 h). Cell death and viability were also analysed (see Supplementary material).

The ability of CHME3 microglia to take up $A\beta_{1-42}$ was confirmed by inspection of living cells using phase contrast/fluorescence microscopy (Fig. 1A), and fluorescence microscopy on the fixed cell suspension used for flow-cytometry (Fig. 1B), and by confocal microscopy on fixed and living cells (Fig. 2). The first signs of uptake (visible in inverted fluorescence microscope) by the microglial cells were observed approximately 4 h after the addition of $A\beta_{1-42}$. Analysis of the intracellular content of $A\beta_{1-42}$ was performed by flow-cytometry at 24 and 96 h after addition of different concentrations of $A\beta_{1-42}$ (Fig. 3A). The uptake increased with increasing concentrations of $A\beta_{1-42}$. A 50-fold increase in the concentration of $A\beta_{1-42}$ from 0.1 to 5 μg/ml resulted in a 10-fold increase in the fraction of cells taking up $A\beta_{1-42}$ ($p < 0.05$).

Effects of $A\beta_{1-42}$ on secretion of BDNF and IL-6

In dose-response experiments with 0.1, 0.5, 1 and 5 μg/ml of $A\beta_{1-42}$, we found that 5 μg/ml reduced the levels of BDNF in the culture medium by 50% compared to vehicle ($p < 0.01$, Fig. 3B). A similar degree of decrease was seen for 5 μg/ml $A\beta_{1-42}$ when compared with the other concentrations of $A\beta_{1-42}$ ($p < 0.01$). At 96 h, the concentrations of 0.1 and 0.5 μg/ml $A\beta_{1-42}$ slightly increased the secretion of BDNF ($p < 0.05$). No effect was seen on the secretion of IL-6 with the concentrations of $A\beta_{1-42}$ tested.

Effects of IL-1β and IFNγ on uptake of $A\beta_{1-42}$

To evaluate the potential of stimulating CHME3 microglia into phagocytosis by inflammation, the cells were incubated with the proinflammatory cytokines IL-1β and IFNγ. Pretreatment with IFNγ resulted in an increase in the proportion of cells showing $A\beta_{1-42}$ uptake ($A\beta_{1-42}$+ cells) with a median increase of 50% at 72 h ($p < 0.01$, Fig. 4, grey boxes), and the combined stimulation with IL-1β and IFNγ lead to a median increase of 66% at 72 h ($p <0.01$) as compared to controls (with $A\beta_{1-42}$ alone), and a 62% increase compared to IL-1β ($p = 0.05$). IL-1β alone had no significant effect on the $A\beta_{1-42}$ uptake. The observed effects appeared to linger at 96 h although the differences were not significant.

Effects of IL-1β and IFNγ on secretion of BDNF and IL-6

The levels of secreted BDNF were markedly reduced by the incubation with the proinflammatory cytokines IL-1β and IFNγ (Fig. 5A, white boxes). Thus, IFNγ decreased the median secretion of BDNF as compared to vehicle with 41% at 72 h and with 44% at 96 h ($p < 0.05$ at both time points). There was no effect of IL-1β alone, but the combination of IL-1β and IFNγ resulted in a significant, almost 50%, decrease in BDNF secretion at 72 and 96 h ($p < 0.05$ at both time points) (Fig. 5A, white boxes).

Treatment of the cells with proinflammatory cytokines resulted in a marked inflammatory response evidenced by a dramatic increase in IL-6 secretion (Fig. 5B, white boxes). At 0 h (*i.e.* 24 h after addition of the cytokines and at the time point of addition of $A\beta_{1-42}$, or in this case, vehicle), IL-1β and IL-1β+IFNγ stimulated the IL-6 secretion, producing levels that were 50 and 90 times higher than control (vehicle), respectively ($p < 0.05$ in both cases (Fig. 5B, white boxes). At 24 h (i.e. after 48 h incubation with the cytokines), IL-1β and IL-1β+IFNγ induced a 200- and 140-fold increase in IL-6 secretion, respectively, and the combination of IFNγ and IL-1β resulted in a 25X higher induction than that by IFNγ alone ($p < 0.05$). The incubation with IL-1β+IFNγ was still effective at 96 h in increasing secreted the levels of IL-6 ($p = 0.0108$). At this time point, IL-1β alone and IFNγ alone significantly increased the levels of IL-6 several-fold ($p < 0.05$ in both cases).

Effects of IL-1β and IFNγ in combination with $A\beta_{1-42}$ on secretion of BDNF and IL-6

Similarly to the effect seen for cytokines, the combined incubation of cytokines and $A\beta_{1-42}$ resulted in reduced secretion of BDNF (Fig. 5A, black boxes). There was no effect of $A\beta_{1-42}$ alone on secretion of BDNF in this series of experiments. At 24 h, pretreatment with IFNγ resulted in a median reduction to 77% in BDNF secretion compared to vehicle (100%) ($p = 0.0086$). At 72 h, the median reduction was 25% ($p < 0.01$) and at 96 h it was 45% ($p < 0.01$) (Fig. 5A, black boxes). The combination of $A\beta_{1-42}$ with IFNγ pretreatment resulted in significantly lower secretion of BDNF than that observed after treatment with $A\beta_{1-42}$ alone at 72 h ($p < 0.01$). Pretreatment with IL-1β decreased the median BDNF level to 88% of vehicle at 96 h ($p < 0.01$). At 72 h, pretreatment with IL-1β+IFNγ resulted in a significant decrease in BDNF level to 64% of vehicle levels ($p < 0.01$) which was also lower than the levels induced by $A\beta_{1-42}$ alone at this time point (97% of vehicle, $p < 0.01$). This effect was still present at 96 h when pretreatment with IL-1β+IFNγ resulted in a decrease in BDNF level to 43% of vehicle

($p < 0.01$), which also at this time point was lower than the level induced by $A\beta_{1-42}$ alone (88% of vehicle, $p = 0.017$). The pretreatment with IL-1β+IFNγ also reduced the levels of BDNF compared with IL-1β at 72 h ($p < 0.05$).

Treatment of the cells with cytokines before addition of $A\beta_{1-42}$ (Fig. 5B, black boxes) resulted in a marked increase in IL-6 secretion that paralleled the increase observed in the absence of $A\beta_{1-42}$ (Fig. 5B, white boxes). In this series of experiments there were no effects of $A\beta_{1-42}$ alone at any time point on the levels of IL-6. The pretreatment of the CHME3 microglial cells with IL-1β or IL-1β+IFNγ had a strong stimulatory effect on the secretion of IL-6 as compared to vehicle and to $A\beta_{1-42}$ alone (Fig. 5B, black boxes). At 24 h, IL-1β increased the median levels of IL-6 25-fold compared with vehicle and $A\beta_{1-42}$ alone ($p < 0.01$ in both cases). A significant stimulatory effect of IL-1β pretreatment was present at 48 h with levels 35-fold those of vehicle and $A\beta_{1-42}$ alone ($p < 0.01$ in both cases). At 48 h, a stimulatory effect of IFNγ became apparent, with median levels of IL-6 that were 5-fold compared with vehicle and $A\beta_{1-42}$ alone ($p < 0.01$ in both cases). Compared with vehicle and $A\beta_{1-42}$ alone, the combined pretreatment with IL-1β+IFNγ induced a 35-fold increase at 24 h ($p < 0.01$ in both cases), a 28-fold increase at 48 h ($p < 0.01$ in both cases) and a 5-fold increase at 96 h compared with vehicle ($p < 0.01$). Also, the combined stimulation with IFNγ and IL-1β prior to $A\beta_{1-42}$ resulted in an increase of almost 6 times in the mean secretion of IL-6 at 48 h as compared to IFNγ alone ($p < 0.005$), but not compared to IL-1β. A significant increase was still seen at 96 h upon pretreatment with the combination of IL-1β and IFNγ, *i.e.* almost 5 times the secretion of IL-6 seen after incubation with vehicle ($p < 0.005$), but no difference in comparison to $A\beta_{1-42}$ alone. Also, at 48 h, the secreted levels of IL-6 following pretreatment with IL-1β+IFNγ were 6 times higher than those seen after pretreatment with IFNγ alone ($p < 0.01$).

Effects of Protollin on uptake of $A\beta_{1-42}$

Pretreatment of the microglial cells with Protollin at 0.001 µg/ml was found to increase the median proportion of cells showing uptake of $A\beta_{1-42}$ to 115% ($p < 0.05$) as compared to control ($A\beta_{1-42}$ alone, 100%, Fig. 4). This effect was seen at 96 h, but no significant changes in any direction could be detected at the earlier time points.

Effects of Protollin, with and without $A\beta_{1\text{-}42}$, on secretion of BDNF and IL-6

Treatment with Protollin alone *i.e.* prior to addition of vehicle, did not affect the levels of BDNF (data not shown). The incubation with $A\beta_{1\text{-}42}$ alone resulted in a decrease in the levels of BDNF in this series of experiments, *i.e.* a reduction to 82% of vehicle at 24 h ($p < 0.0000001$), to 77% of vehicle at 48 h ($p < 0.005$) and to 95% of vehicle at 96 h ($p < 0.05$). In cultures pretreated with the lowest concentration of Protollin (0.001 µg/ml) followed by incubation with $A\beta_{1\text{-}42}$ there was a 22% reduction at the 24 h time point as compared to vehicle ($p = 0.05$), whereas at the higher concentrations of Protollin and at later time points there was no significant differences in the secretion of BDNF in comparison with control conditions (no Protollin and no $A\beta_{1\text{-}42}$).

Pretreatment with Protollin followed by $A\beta_{1\text{-}42}$ or vehicle did not induce any significant effects on the levels of IL-6 in culture supernatants (data not shown).

Cellular markers and relation to phagocytosis of $A\beta_{1\text{-}42}$

The uptake of $A\beta_{1\text{-}42}$ in cells expressing the inflammatory markers IL-1β, IL-1RI and iNOS in the cultures treated with $A\beta_{1\text{-}42}$ following pretreatment with Protollin was demonstrated by immunocytochemistry (Fig. 6). The proportion of cells positive for these markers was analysed by flow-cytometry, both after incubation with $A\beta_{1\text{-}42}$ alone, and after pretreatment with Protollin, in order to assess the phenotype of the cells taking up $A\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$+) (Fig. 7).

IL-1β immunoreactive cells

In untreated (vehicle) cultures, the proportion of CHME3 microglial cells with immunoreactivity to IL-1β had a median of 2.9% at 24 h. Incubation of the cells with 1 µg/ml $A\beta_{1\text{-}42}$ did not significantly affect the number of IL-1β immunoreactive cells compared with vehicle at any time point.

The expression of IL-1β in $A\beta_{1\text{-}42}$+ cells was not significantly different from that in $A\beta_{1\text{-}42}$- cells according to the Wilcoxon Matched Pairs test, except at 72 h when the population of $A\beta_{1\text{-}42}$+/IL-1β+ cells was larger than the $A\beta_{1\text{-}42}$-/IL-1β+ cell population (Fig. 7A).

Pretreatment with Protollin increased the number of IL-1β+ cells displaying $A\beta_{1\text{-}42}$ uptake (Fig 7A). Significant differences were observed between $A\beta_{1\text{-}42}$+/IL-1β+ cells and $A\beta_{1\text{-}42}$-/IL-1β+ cells treated with Protollin at 24 h in concentrations of 0.001 µg/ml ($p < 0.05$), 0.1 µg/ml

($p < 0.05$) and 1 µg/ml ($p < 0.05$), at 48 h in concentrations of 0.1 µg/ml ($p < 0.05$) and 1 µg/ml ($p < 0.05$), and at 72 h in a concentration of 0.1 µg/ml ($p < 0.05$).

IL-1RI immunoreactive cells

In untreated (vehicle) cultures the proportion of CHME3 microglial cells with immunoreactivity for IL-1RI (IL-1RI+) had a median value of 12.5% at 24 h. Incubation with 1 µg/ml $A\beta_{1-42}$, with or without pretreatment with Protollin, did not significantly affect the number of IL-1RI immunoreactive cells at any time point (Fig 7B).

When incubated with $A\beta_{1-42}$ alone, the population of $A\beta_{1-42}$+/IL-1RI+ cells was significantly larger than the $A\beta_{1-42}$-/IL-1RI+ population at 24, 48 and 72 h ($p < 0.05$, Fig 7B). When pretreated with Protollin, there was a significantly larger proportion of $A\beta_{1-42}$+ cells that were immunoreactive to IL-1RI ($A\beta_{1-42}$+/IL-1RI+) at 24 h in concentrations of 0.001 µg/ml ($p < 0.05$), 0.1 µg/ml ($p < 0.05$) and 1 µg/ml ($p < 0.05$), at 48 h in concentrations of 0.1 µg/ml ($p < 0.05$) and 1 µg/ml ($p < 0.05$) and at 72 h in a concentration of 0.1 µg/ml ($p < 0.05$).

iNOS immunoreactive cells

In untreated (vehicle) cultures the median proportion of CHME3 microglial cells with immunoreactivity to iNOS (iNOS+) had a median of 16% at 24 h under basal (vehicle) conditions. None of the treatments affected the total number of cells immunoreactive for iNOS.

When incubated with $A\beta_{1-42}$ alone, the population of $A\beta_{1-42}$+/iNOS+ cells was significantly larger than the population of $A\beta_{1-42}$-/iNOS+ cells at all time points ($p = 0.0173$ at 24 h, $p = 0.0172$ at 48 h, $p = 0.0117$ at 72 h and $p = 0.05$ at 96 h, Fig 7C). Also when cells were pretreated with Protollin, the $A\beta_{1-42}$+/iNOS+ population was significantly larger than the $A\beta_{1-42}$-/iNOS+ population, at all concentrations tested (0.001 µg/ml: $p = 0.0117$ at 24 h, $p = 0.0357$ at 48 h and $p = 0.025$ at 72 h; 0.01 µg/ml: $p = 0.0117$ at 24 h and $p = 0.0117$ at 48 h; 0.1 µg/ml: $p = 0.0117$ at 24 h, $p = 0.05$ at 48 h and $p = 0.0251$ at 72 h: and $p = 0.025$ at 72 h: 0.1 µg/ml: $p = 0.0117$ at 24 h, Fig 7C).

Correlation between secretion of BDNF and uptake of $A\beta_{1-42}$

We found a significant negative correlation ($p < 0.05$) between the levels of BDNF in culture supernatant and the proportion of $A\beta_{1-42}$+ cells at all the time points in the experiments (Fig.

8). The data were analysed by correlating the BDNF-levels with the proportion of $A\beta_{1-42}$+ cells in all the treatments at one time point, and in one treatment at one time point, using the Spearman Rank Order Correlation test. There was a significant negative correlation in all analyses performed, except at 48 h, when the treatment with 0.001 mg/ml Protollin was void of a significant correlation.

Cell death and viability

The effects of $A\beta_{1-42}$ and the immunomodulatory substances on cell death and viability were analysed by the LDH and MTT assays, respectively. Neither $A\beta_{1-42}$ nor Protollin, nor their combination, produced any significant effects on cell viability (data not shown).

The incubation with IFNγ or IL-1β+IFNγ decreased the signal from the MTT assay significantly to almost 50% of vehicle starting at 48 h ($p < 0.05$), and at 72 ($p < 0.005$) and 96 h ($p < 0.005$) (Fig. 9). This effect was seen both when the microglia were stimulated with the cytokines alone and when they were added before $A\beta_{1-42}$.

There were no significant differences in the LDH-activity in the medium from any of the treatments (not shown). Inspection with microscope, however, showed signs of cell death in treatments associated with a significant decrease in MTT signal.

Discussion

In this study, a human microglial cell line was used to model phagocytosis of Aβ and to evaluate the effects of different substances. The ability to analyse the effects of different substances with a human microglial cell line, which can be cultured in significant quantities, and the cellular reactions to pathological factors, such as Aβ, is a valuable tool in studies of human CNS-pathologies. The CHME3 cell line was established by Prof. M. Tardieu [37]. This cell line responds to stimulation with LPS by increased secretion of IL-6 [38], a cytokine also secreted under basal conditions. We have found that CHME3 microglial cells also secrete low, but detectable, levels of IL-1β and TNFα as well as other cytokines (unpublished observations).

Phagocytosis of $A\beta_{1-42}$ was established using fluorophore-labelled $A\beta_{1-42}$. The uptake was first detectable at 4 h after the addition as seen by microscopical analysis of living cells. Phagocytosis of $A\beta_{1-42}$ was differentiated from unspecific adherence to cell membranes by confocal microscopy which confirmed the lysosomal location of $A\beta_{1-42}$. In terms of

inflammatory response, the incubation with $A\beta_{1-42}$ at the concentration used (1 μg/ml) did not affect the basal secretion of IL-6, in accordance with studies on primary human embryonic microglia in which $A\beta_{1-42}$ failed to increase transcription of the IL-6 gene and $A\beta_{25-35}$ had no effect on IL-6 secretion [39]. Similarly, the secretion of IL-6 from rat microglial cells was not altered upon incubation with 75 μM $A\beta_{1-42}$, a concentration 150-fold the concentration used in this study [18]. In contrast, primary mouse microglia have been shown to respond with markedly increased IL-6 secretion upon stimulation with $A\beta_{1-42}$ in a concentration similar to that in the present study [40], indicating a species difference. In our previous studies on the CHME3 microglia a marked increase in the secreted levels of IL-6 could be seen upon incubation with $A\beta_{1-40}$ [38], suggesting a difference in the immune-activating properties of the two forms of Aβ.

We show here that the human microglial cell line produces and secretes BDNF, in agreement with studies on human *post mortem* tissue [41]. Studies in different injury models in animals have shown the induction of BDNF production in microglia [42], supporting a view of BDNF as a glial response to neuronal injury serving to help neurons to recover. In this context it is remarkable that the incubation with $A\beta_{1-42}$ reduced the secretion of BDNF, which to our knowledge is the first time $A\beta_{1-42}$ has been shown to exert this effect on any cell type. This adds to the negative effects of $A\beta_{1-42}$ and indicates a further reason for limiting the amyloidosis in AD. Evidence for detrimental interference by $A\beta_{1-42}$ on BDNF signalling in neurons has been provided previously [43]. A reduced secretion and an impaired signalling of BDNF may contribute to the cell death and impaired neuronal function in AD. In fact, decreased levels of BDNF have been observed in the CSF of AD patients, and at later stages of the disease this reduction correlated with the severity of impairment [44].

Interestingly, a marked decrease in basal BDNF secretion was observed also upon incubation of the microglial cells with IFNγ, an effect that was also observed upon co-incubation with IL-1β. In contrast, studies on rodent microglia showed an increase in the levels of BDNF in association with induction of inflammation [42], again suggesting species differences in microglial responses. Furthermore, studies on rat astrocytes indicated a stimulating effect on BDNF production by TNFα mediated by NFκB [45]. In this study IL-1β, which is also known to activate NFκB [46], caused a mild inhibitory effect on BDNF secretion at 96 h.

To analyse the possibility to stimulate the phagocytosis of $A\beta_{1-42}$, we analysed the effects of different immunomodulatory substances. Pretreatment of the human microglial cells with

IFNγ, alone or together with IL-1β, resulted in a significant increase in the proportion of cells with an uptake of $A\beta_{1-42}$. This was accompanied by a pronounced reduction in BDNF secretion, as well as decreased cell viability. The results indicate that pro-inflammatory factors may be able to stimulate $A\beta_{1-42}$ phagocytosis. However, the stimulation by IFNγ was seen in the later time points and it may be speculated that the acute inflammation at the start of the experiment had expired at this stage with presumably only low levels of the added cytokines still present in the medium. The effects observed may thus be due to factors induced by, and secondary to IFNγ. The effect of long-term, chronic exposure to inflammatory stimulation may be different. The influence of inflammation on $A\beta_{1-42}$ phagocytosis and clearance is a complex matter, where factors such as age and species may be pivotal. Even more complexity is added by the effects of $A\beta_{1-42}$ itself on inflammation, including the findings that the 40 and 42 amino acid peptides, and the aggregational form of $A\beta_{1-42}$ (monomers, oligomers, protofibrils, etc), induces different inflammatory responses as seen in studies on rat microglia [18]. Furthermore, the shorter and longer species of $A\beta_{1-42}$ have not been characterized with regard to their influence on glia. In the present study we prepared $A\beta_{1-42}$ by dissolving the lyophilized peptide in pure DMSO, and therefore it is reasonable to assume that soluble monomers or oligomers dominate at the start of the experiments. Interestingly, we could not detect any significant difference in the proportion of $A\beta_{1-42}$+ cells with time (data not shown). Factors influencing this proportion may be the rate of phagocytosis, degradation of the peptide, or changes in cell number. Our data show an ongoing cell proliferation continuing to the end of the experiment. This fact, taken together with a stable $A\beta_{1-42}$+ cell proportion with time suggest that phagocytosis is continuous. An exception was seen upon treatment with IFNγ, in which the $A\beta_{1-42}$+ proportion increased significantly simultaneous with a reduction in cell viability.

Protollin, a proteosome-based adjuvant with immunomodulatory activities, was found to modestly increase $A\beta_{1-42}$ uptake by the human CHME3 microglia at 96 h after addition of $A\beta_{1-42}$, but not at the earlier time points and only at the lowest concentration tested (0.001 µg/ml). This potentially stimulatory effect of Protollin on $A\beta_{1-42}$ uptake is concordant with results from *in vivo* studies on Protollin in an AD mouse model [34], showing clearance of amyloid plaques and improved cognitive performance upon intranasal administration of Protollin. The limited effect of Protollin in the present *in vitro* experiments as compared to the

in vivo studies may have several explanations. In the mouse *in vivo* model, the stimulatory effect of Protollin on Aβ-uptake appears to be mediated via activation of monocytes in the periphery that migrate to the brain and phagocytose Aβ, rather than direct activation of microglial cells resident in the brain [34]. Also, there was no evidence from the *in vivo* studies that Protollin translocates to the brain following nasal administration [34]. The modest stimulatory effect of Protollin on $A\beta_{1-42}$ uptake by microglial cells in the present study may reflect the absence of accessory cells in the *in vitro* cultures, *i.e.* cells that Protollin directly activates in the periphery *in vivo*. Alternatively, considering that the effect of Protollin did not become apparent until the later part of the experiment, it may also be speculated that activation of microglial cell precursors by Protollin in the periphery results in the secretion of factors in a para/autocrine fashion, that with time build up to concentrations that stimulate phagocytosis. Species differences should also be considered. The response repertoire of glial cells appear to be different in human as compared with murine cells [47] [48].
Interestingly, the pretreatment with Protollin appeared to ameliorate the $A\beta_{1-42}$-induced decrease in BDNF secretion. This highlights a therapeutic target: stimulation of glial cells for the production of beneficial and neuroprotective molecules. As indicated above, beneficial effects of Protollin in the context of immunotherapy may be elicited through stimulation of peripheral monocytes [34], directly, or indirectly through interaction with other immunocompetent cells. The present data, *e.g.* on BDNF, suggest that Protollin or similarly acting substances may also stimulate beneficial effects on glial cells within the brain.
To our knowledge, no studies on human cells have until now investigated the effects of TLR2 activation on BDNF secretion.
Analysis with flow-cytometry showed that cells displaying phagocytosis of $A\beta_{1-42}$ had a significantly higher degree of expression of IL-1RI and iNOS, indicating that phagocytosis of $A\beta_{1-42}$ was associated with an inflammatory phenotype. Similarly, in a mouse AD-model, the expression of CD11b, a microglial activation marker that has been used as an indicator of harmful inflammation in several studies, was associated with removal of Aβ [34].

We also observed a strong negative correlation between the $A\beta_{1-42}$+ cell proportion and the levels of BDNF. This result can be due to a negative effect on phagocytosis by BDNF or a decrease in BDNF secretion by cells performing phagocytosis. In a previous study BDNF was found to stimulate phagocytosis [49]. Although the experiments were performed on mouse

peritoneal macrophages the results support the latter explanation for the negative correlation between BDNF levels and phagocytosis.

Protollin appeared to decrease the proportion of iNOS+ cells displaying phagocytosis of $A\beta_{1-42}$, although the total number of iNOS+ cells remained unchanged. This proportion was significantly higher at all time points when incubated with $A\beta_{1-42}$ alone. At 96 h, the pre-treatment with Protollin abolished this difference. A reduction in the levels of iNOS is beneficial due to the contribution of this enzyme to oxidative stress, supporting beneficial effects of Protollin.

The decrease in cell viability accompanying the induction of Aβ phagocytosis by IFNγ and IL-1β suggests microglial cell death. This was confirmed by microscopical inspection of the cultures. In spite of this, there was no detectable increase in the LDH-activity. However, a decrease in cell number as indicated by the MTT-assay could mask an increase in cell death as measured by the LDH-assay since fewer cells are available to release LDH into the medium. IFNγ has been shown to induce cell death in murine microglia, concomittant with an upregulation of the expression of Fas and FasL [50]. In addition, IL-1β and IFNγ are known to be involved in the expression and activation of iNOS [51,52], which in turn may lead to oxidative stress.

In conclusion, we provide a model suitable for testing candidates for stimulating the phagocytosis of $A\beta_{1-42}$ by human microglial cells. The capacity to withstand serum-withdrawal for long periods of time and the high rate of proliferation makes the human CHME3 cell line suitable for this type of studies. Expanding primary cultures of microglia for large experimental series is not trivial. The data presented indicate differences between human microglial cells and murine glia, as described in other studies. In the present study, we show that the immunomodulatory substance Protollin affects the proportion of cells phagocytosing Aβ and their expression of inflammatory markers. Pretreatment with IFNγ had a robust stimulatory effect on phagocytosis of $A\beta_{1-42}$ at later time points, suggesting influence of secondary factors induced by this cytokine. In addition, we present data suggesting a neuropathological role for IFNγ by decreasing BDNF levels. Importantly, we show a strong negative effect of $A\beta_{1-42}$ on BDNF secretion. The presumed decrease in secretion of BDNF from phagocytic cells, indicated by the negative correlation between phagocytosis and BDNF, suggests an interesting parameter for future evaluation of potential drugs. The results present

a scenario in which inflammation increases phagocytosis of $A\beta_{1-42}$, induces microglial cell death and reduces secretion of BDNF. This reduction is unwanted, since it deprives the brain of an important neuroprotective and plasticity-promoting factor. Considering the presence of inflammation in AD, it may be suggested that the combined effect of Aβ, IL-1β and IFNγ on the secretion of BDNF from microglia may contribute to the neuronal pathology in AD. We hope that this and future studies can help develop directed and controlled activation of differential pro- and anti-inflammatory responses for therapeutic use.

Acknowledgements

The support of this work by grants from the Swedish Research Council (072194), Swedish Brain Power, The Knut and Alice Wallenberg Foundation, Karolinska Institutet Research funds, Stiftelsen för Gamla Tjänarinnor, Alzheimer Foundation Sweden, Gun och Bertil Stohnes stiftelse, and Stockholm County Council, are gratefully acknowledged. The input and suggestions from Glaxo-Smith Kline Biologicals are also acknowledged.

References

1. Lee VM, Biomedicine. Tauists and β-aptists united - well almost!, *Science*, 293:1446-1447, 2001
2. Hardy J, Selkoe DJ, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, *Science*, 297:353-356, 2002
3. Guntert A, Dobeli H, Bohrmann B, High sensitivity analysis of amyloid-β peptide composition in amyloid deposits from human and PS2APP mouse brain, *Neuroscience*, 143:461-475, 2006
4. Kuo YM, Emmerling MR, Vigo-Pelfrey C, Kasunic TC, Kirkpatrick JB, Murdoch GH, Ball MJ, Roher AE, Water-soluble Aβ (N-40, N-42) oligomers in normal and Alzheimer disease brains, *Journal of Biological Chemistry*, 271:4077-4081, 1996
5. Deitmer JW, Strategies for metabolic exchange between glial cells and neurons, *Respiratroy Physiology*, 129:71-81, 2001
6. Noske W, Lentzen H, Lange K, Keller K, Phagocytotic activity of glial cells in culture, *Experimental Cell Research*, 142:437-445, 1982
7. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmeling M, Fiebich BL, Finch CE, Frautschy S, Griffin WST, Hampbell H, Hüll M, Landreth G, Lue L-F, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen J, Streit W, Strohmeyer R, Tooyama I, Muiswinkel FLW, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyess-Coray T, Inflammation and Alzheimer's disease, *Neurobiology of Aging*, 21:383-421, 2000
8. McGeer EG, McGeer PL, Inflammatory processes in Alzheimer's disease, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 27:741-749, 2003
9. Ide CF, Scripter JL, Coltman BW, Dotson RS, Snyder DC, Jelaso A, Cellular and molecular correlates to plasticity during recovery from injury in the developing mammalian brain, *Progress in Brain Research*, 108:365-377, 1996
10. Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D, Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease, *Journal of Neuroimmunology*, 24:173-182., 1989
11. Griffin WST, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, White III CL, Araoz C, Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease, *The Proceedings of the National Academy of Sciences*, 86:7611-7615, 1989
12. Huell M, Strauss S, Volk B, Berger M, Bauer J, Interleukin-6 is present in early stages of plaque formation and is restricted to the brains of Alzheimer's disease patients, *Acta Neuropathology*, 89:544-551, 1995
13. Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H, Riederer P, Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients, *Neuroscience Letters*, 202:17-20, 1995
14. Cacabelos R, Barquero M, Garcia P, Alvarez XA, Varela de Seijas E, Cerebrospinal fluid interleukin-1β (IL-1β) in Alzheimer's disease and neurological disorders, *Methods and Findings in Experimental and Clinical Pharmacology*, 13:455-458, 1991
15. Araujo DM, Cotman CW, β-amyloid stimulates glial cells in vitro to produce growth factors that accumulate in senile plaques in Alzheimer's disease, *Brain Research*, 569:141-145, 1992

16. Benveniste EN, Nguyen VT, O'Keefe GM, Immunological aspects of microglia: relevance to Alzheimer's disease, *Neurochemistry International*, 39:381-391., 2001
17. Garcao P, Oliveira CR, Agostinho P, Comparative study of microglia activation induced by amyloid-β and prion peptides: role in neurodegeneration, *Journal of Neuroscience Research*, 84:182-193, 2006
18. Lindberg C, Bardyl Selenica M-L, Westlind-Danielsson A, Schultzberg M, β-amyloid protein structure determines the nature of cytokine release from rat microglia, *Journal of Molecular Neuroscience*, 27:1-12, 2005
19. Selkoe DJ, Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease, *Annual Review of Cell and Developmental Biology*, 10:373-403, 1994
20. Rogers JT, Leiter LM, McPhee J, Cahill CM, Zhan SS, Potter H, Nilsson LN, Translation of the alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5'-untranslated region sequences, *Journal of Biological Chemistry*, 274:6421-6431, 1999
21. Grilli M, Ribola M, Alberici A, Valerio A, Memo M, Spano P, Identification and characterization of a κB/Rel binding site in the regulatory region of the amyloid precursor protein gene, *Journal of Biological Chemistry*, 270:26774-26777, 1995
22. Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B, Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of Aβ1-40 and Aβ1-42 by human astrocytes, *Neurobiological Disorders*, 7:682-289, 2000
23. Mrak RE, Griffin WS, Interleukin-1, neuroinflammation, and Alzheimer's disease, *Neurobiology of Aging*, 22:903-908., 2001
24. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P, Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse, *Nature*, 400:173-177, 1999
25. Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C, Subacute meningoencephalitis in a subset of patients with AD after $A\beta_{1-42}$ immunization, *Neurology*, 61:46-54, 2003
26. von Bernhardi R, Ramirez G, Toro R, Eugenin J, Pro-inflammatory conditions promote neuronal damage mediated by amyloid precursor protein and decrease its phagocytosis and degradation by microglial cells in culture, *Neurobiological Disorders*, 26:153-164, 2007
27. Koenigsknecht-Talboo J, Landreth GE, Microglial phagocytosis induced by fibrillar β-amyloid and IgGs are differentially regulated by proinflammatory cytokines, *Journal of Neuroscience*, 7:8240-8249, 2005
28. Bartfai T, Sanchez-Alavez M, Andell-Jonsson S, Schultzberg M, Vezzani A, Danielsson E, Conti B, Interleukin-1 system in CNS stress: seizures, fever, and neurotrauma, *Annals of the New York Academy of Sciences*, 1113:173-177, 2007
29. Beck T, Lindholm D, Castren E, Wree A, Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus, *Journal of Cerebral Blood Flow & Metabolism*, 14:689-692, 1994
30. Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF, The role of brain-derived neurotrophic factor receptors in the mature

hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB, *Journal of Neuroscience*, 20:6888-6897, 2000
31. Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH, Safety and immunogenicity of a proteosome-Shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults, *Infection and Immunity*, 69:4545-4553, 2001
32. Chabot SM, Chernin TS, Shawi M, Wagner J, Farrant S, Burt DS, Cyr S, Neutra MR, TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces, *Vaccine*, 25:5348-5358, 2007
33. Boivin A, Pineau I, Barrette B, Filali M, Vallieres N, Rivest S, Lacroix S, Toll-like receptor signaling is critical for Wallerian degeneration and functional recovery after peripheral nerve injury, *Journal of Neuroscience*, 27:12565-12576, 2007
34. Frenkel D, Puckett L, Petrovic S, Xia W, Chen G, Vega J, Dembinsky-Vaknin A, Shen J, Plante M, Burt DS, Weiner HL, A nasal proteosome adjuvant activates microglia and prevents amyloid deposition, *Annals of Neurology*, 63:591-601, 2008
35. Samdani AF, Dawson TM, Dawson VL, Nitric oxide synthase in models of focal ischemia, *Stroke*, 28:1283-1288, 1997
36. Lee SC, Zhao ML, Hirano A, Dickson DW, Inducible nitric oxide synthase immunoreactivity in the Alzheimer disease hippocampus: association with Hirano bodies, neurofibrillary tangles, and senile plaques, *Journal of Neuropathology & Experimental Neurology*, 58:1163-1169, 1999
37. Janabi N, Peudenier S, Heron B, Ng KH, Tardieu M, Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen, *Neurosci Lett*, 195:105-108, 1995
38. Lindberg C, Hjorth E, Post C, Winblad B, Schultzberg M, Cytokine production by a human microglial cell line: effects of β-amyloid and α-melanocyte-stimulating hormone, *Neurotoxicity Research* 8:267-276, 2005
39. Lee YB, Nagai A, Kim SU, Cytokines, chemokines, and cytokine receptors in human microglia, *Journal of Neuroscience Research*, 69:94-103., 2002
40. Szczepanik AM, Funes S, Petko W, Ringheim GE, IL-4, IL-10 and IL-13 modulate $A\beta_{1-42}$-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line, *Journal of Neuroimmunology*, 113:49-62, 2001
41. Knott C, Stern G, Kingsbury A, Welcher AA, Wilkin GP, Elevated glial brain-derived neurotrophic factor in Parkinson's diseased nigra, *Parkinsonism and Related Disorders*, 8:329-341, 2002
42. Lai AY, Todd KG, Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury, *Glia*, 56:259-270, 2008
43. Tong L, Balazs R, Thornton PL, Cotman CW, β-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons, *Journal of Neuroscience*, 24:6799-6809, 2004
44. Laske C, Stransky E, Leyhe T, Eschweiler GW, Maetzler W, Wittorf A, Soekadar S, Richartz E, Koehler N, Bartels M, Buchkremer G, Schott K, BDNF serum and CSF concentrations in Alzheimer's disease, normal pressure hydrocephalus and healthy controls, *Journal of Psychiatric Research*, 41:387-394, 2007
45. Saha RN, Liu X, Pahan K, Up-regulation of BDNF in astrocytes by TNF-α: a case for the neuroprotective role of cytokine, *Journal of Neuroimmune Pharmacology*, 1:212-222, 2006

46. Shirakawa F, Chedid M, Suttles J, Pollok BA, Mizel SB, Interleukin 1 and cyclic AMP induce kappa immunoglobulin light-chain expression via activation of an NF-κB-like DNA-binding protein, *Molecular & Cellular Biology*, 9:959-964, 1989
47. Colton C, Wilt S, Gilbert D, Chernyshev O, Snell J, Dubois-Dalcq M, Species differences in the generation of reactive oxygen species by microglia, *Molecular and Chemical Neuropathology*, 28:15-20, 1996
48. Lee SC, Brosnan CF, Cytokine regulation of iNOS expression in human glial cells, *Methods*, 10:31-37, 1996
49. Asami T, Ito T, Fukumitsu H, Nomoto H, Furukawa Y, Furukawa S, Autocrine activation of cultured macrophages by brain-derived neurotrophic factor, *Biochemical & Biophysical Research Communications*, 344:941-947, 2006
50. Badie B, Schartner J, Vorpahl J, Preston K, Interferon-γ induces apoptosis and augments the expression of Fas and Fas ligand by microglia in vitro, *Experimental Neurology*, 162:290-296, 2000
51. Akama KT, Van Eldik LJ, β-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1β- and tumor necrosis factor-α (TNFα)-dependent, and involves a TNFα receptor-associated factor- and NFκB-inducing kinase-dependent signaling mechanism, *Journal of Biological Chemistry*, 275:7918-7924., 2000
52. Kim H, Lee E, Shin T, Chung C, An N, Inhibition of the induction of the inducible nitric oxide synthase in murine brain microglial cells by sodium salicylate, *Immunology*, 95:389-394, 1998

Figure Legends

Fig. 1 A – B. Uptake of $A\beta_{1-42}$ in human microglial cells. The micrographs in A show living human CHME3 microglial cells in culture after incubation with $A\beta_{1-42}$, seen by phase contrast and fluorescence microscopy, and after merging of these. The micrographs in B show fixed CHME3 microglial cells in suspension after incubation with $A\beta_{1-42}$, seen by fluorescence microscopy with filters for propidium iodide (PI) staining and HiLyte488-conjugated $A\beta_{1-42}$, respctively, and the micrographs are merged in the 3rd micrograph. Magnifications 10X (A) and 40X (B).

Fig. 2. Uptake of $A\beta_{1-42}$ in human microglial cells. The confocal micrograph (63X) shows fixed CHME3 microglial cells demonstrating intracellular lysosomal location of HiLyte488-conjugated $A\beta_{1-42}$ (green filter). Lysosomes were visualized by staining with an antibody against lysosome-associated membrane protein-2 (lamp-2) and Cy3-conjugated secondary antibodies (red filter). Yellow colour thus indicates colocalization of lamp-2 and HiLyte488-conjugated $A\beta_{1-42}$.

Fig. 3A - B. Uptake of $A\beta_{1-42}$ (A) and the concurrent secretion of interleukin-6 (IL-6) and brain-derived neurotrophic factor (BDNF) (B) by human CHME3 microglial cells at 24 and 96 h. The cells were incubated with concentrations of $A\beta_{1-42}$, ranging between 0.1 and 5 μg/ml, or vehicle. The cells were harvested and the medium collected at 24 and 96 h. In A, the data are expressed as the proportion of cells with intracellular $A\beta_{1-42}$. n = 4. In B, the data are expressed as % of control (vehicle) set at 100% and shown as median ± percentiles (25% - 75% and 10% - 90%). There was a significant effect of the treatment on the uptake of $A\beta_{1-42}$ at 24 h ($p = 0.0045$) and at 96 h ($p = 0.0375$). The secretion of BDNF was also significantly altered by the treatment at 24 h ($p = 0.0102$) and at 96 h ($p = 0.0375$). Statistical difference from $A\beta_{1-42}$, 5 μg/ml, is indicated by ¤¤ ($p < 0.01$), statistical difference from vehicle is indicated by ** ($p < 0.01$).

Fig. 4. Effects of Protollin (white boxes) and, interleukin-1β (IL-1β) and interferon-γ (IFNγ) (grey boxes) on the uptake of $A\beta_{1-42}$ in human CHME3 microglial cells. The cells were incubated with 1 μg/ml $A\beta_{1-42}$ following pre-stimulation for 24 h with Protollin at 0.001 – 1

µg/ml, or 50 ng/ml IL-1β and 50 ng/ml IFNγ. The cells were harvested at 24, 48, 72 and 96 h after addition of $A\beta_{1-42}$. The data are expressed as % uptake of control $A\beta_{1-42}$ set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). n = 19 (Protollin) or n = 4 (cytokines) for 24 and 48 h, n = 17 (Protollin) of n = 6 (cytokines) for 72 and 96 h. A statistically significant effect of treatment was found by Kruskal-Wallis ANOVA at 96 h when incubating microglia with Protollin ($p = 0.0157$) and at 72 h when incubating with IL-1β and/or IFNγ ($p = 0.0022$). Statistical difference from control is indicated by # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.005$). † indicates statistical difference ($p < 0.01$) between IL-1β and IL-1β+IFNγ.

Fig. 5A - B. Effects of incubation with interleukin-1β (IL-1β) and interferon-γ (IFNγ), added 24 h before incubation with 1 µg/ml $A\beta_{1-42}$ (black boxes) HiLyte488-conjugated $A\beta_{1-42}$ or vehicle (white boxes), on secreted levels of IL-6 (A) and BDNF (B) from CHME3 microglial cells. The cells were incubated with 1 µg/ml HiLyte488-conjugated $A\beta_{1-42}$ following pre-stimulation for 24 h with 50 ng/ml IL-1β and 50 ng/ml IFNγ. The cells were harvested at 24, 48, 72 and 96 h after addition of $A\beta_{1-42}$. The data are expressed as % uptake of control vehicle set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). n = 4 for 24 h and 48 h and n = 6 for 72 and 96 h. A statistically significant effect of treatment was found by Kruskal-Wallis ANOVA at 24 h ($p = 0.0087$), at 72 h ($p = 0.004$) and at 96 h ($p = 0.004$). Statistical difference from control is indicated by ** ($p < 0.01$), difference from $A\beta_{1-42}$ # is indicated by ($p < 0.05$) and ## ($p < 0.01$). † indicates statistical difference ($p < 0.05$) between IL-1β and IL-1β+IFNγ.

Fig. 6. Uptake of $A\beta_{1-42}$ and expression of cellular markers in human microglial cells. The micrographs show human CHME3 microglial cells after incubation in culture with biotinylated $A\beta_{1-42}$, after which they were fixed and stained with antibodies against interleukin-1β (IL-1β), IL-1 receptor type I (IL-1RI) and inducible nitric oxide synthase (iNOS), and subsequent incubation with Cy2-conjugated secondary antibodies. Cell nuclei were stained with propidium iodide (PI) and the biotinylated $A\beta_{1-42}$ was visualized with AMCA-conjugated streptavidin. All micrographs are in 20X magnification.

Fig. 7A - C. Differential phenotype of human CHME3 microglia, in the populations of cells displaying ($A\beta_{1-42}$+, grey boxes) or not displaying ($A\beta_{1-42}$-, white boxes) phagocytosis of $A\beta_{1-42}$, with regard to immunoreactivity for interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and IL-1β receptor type I (IL-1RI), following pretreatment with Protollin (0.001 – 1 µg/ml). After 24 – 96 h of exposure to $A\beta_{1-42}$, the cells were subjected to immunocytochemistry and analysed by flow-cytometry. The population of cells with immunoreactivity to the different markers within the $A\beta_{1-42}$+ cell population was compared with the corresponding population in the $A\beta_{1-42}$- cell population in each treatment group, using the Wilcoxon Matched Pairs Test. The data are shown as median ± percentiles (25% - 75% and 10% - 90%), n =7. Statistical differences between the $A\beta_{1-42}$+ and $A\beta_{1-42}$- cells with regard to each marker are indicated by * ($p < 0.05$).

Fig. 8. Correlation between the level of BDNF and the proportion of $A\beta_{1-42}$+ cells. The levels of BDNF (pg/ml) and the proportion of $A\beta_{1-42}$+ cells in the Protollin series of experiments were analysed at each time point using the Spearmen Rank Order Correlations test. A significant negative correlation was found at all time points. A statistical significant correlation is indicated by * ($p < 0.05$).

Fig. 9. . Effects of interleukin-1β (IL-1β) and interferon-γ (IFNγ) on cell viability in human CHME3 microglial cells treated with $A\beta_{1-42}$ (grey boxes) or vehicle (white boxes). The cells were pre-incubated with 50 ng/ml IL-1β and 50 ng/ml IFNγ for 24 h prior to addition of $A\beta_{1-42}$ (1 µg/ml). At 24, 48, 72 and 96 h after addition of $A\beta_{1-42}$ the viability of the cultures were assessed with the MTT assay. The data are expressed as % of control (vehicle) set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). n = 4 (24, 48 h), n = 6 (72, 96 h). Statistical difference from vehicle is indicated by * ($p < 0.05$), and *** ($p < 0.005$), and statistical difference from $A\beta_{1-42}$ is indicated by # ($p < 0.05$) and ## ($p < 0.01$). ǂ ($p < 0.01$) indicates statistical difference between IFNγ and IL-1β+IFNγ, and † ($p < 0.05$) and ††† ($p < 0.05$) indicate differences between IL-1β and IL-1β+IFNγ.

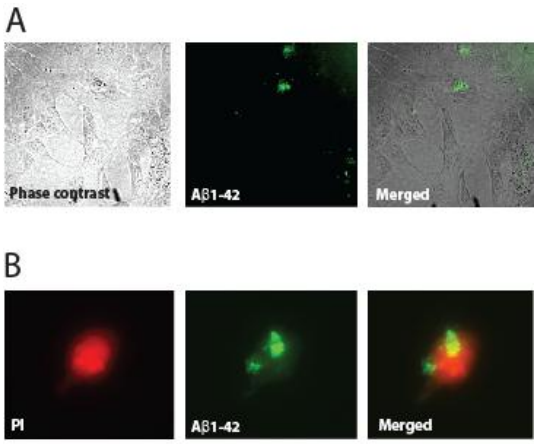

Fig. 1 A - B
Hjorth et al, 2009

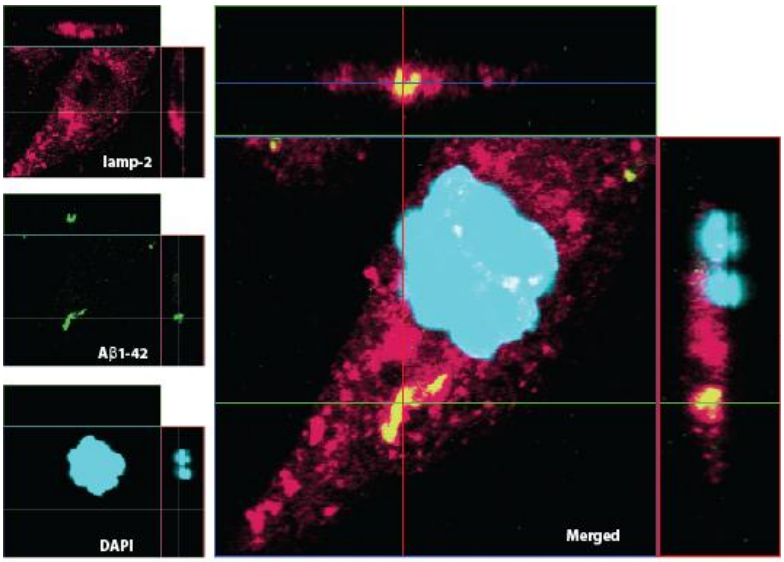

Fig. 2
Hjorth et al, 2009

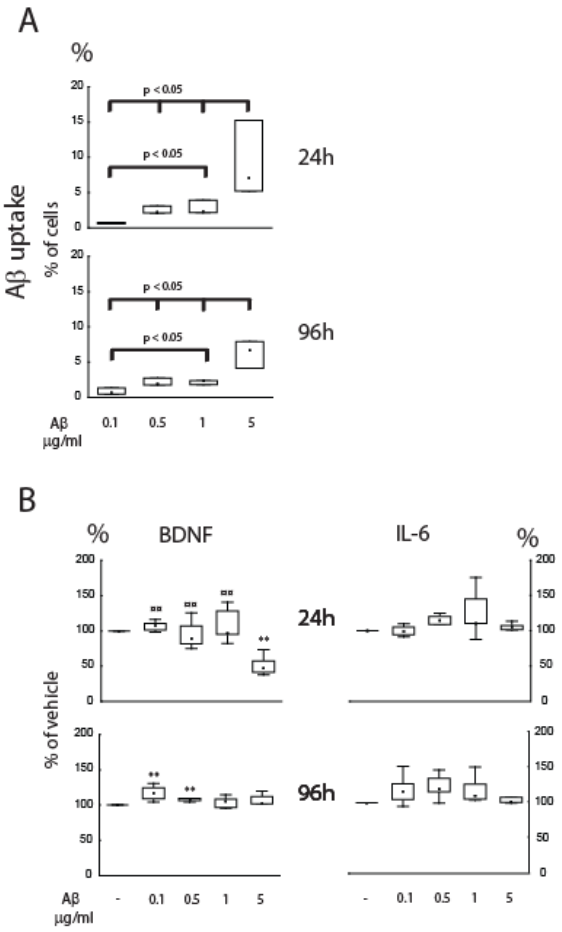

Fig. 3 A - B
Hjorth et al, 2009

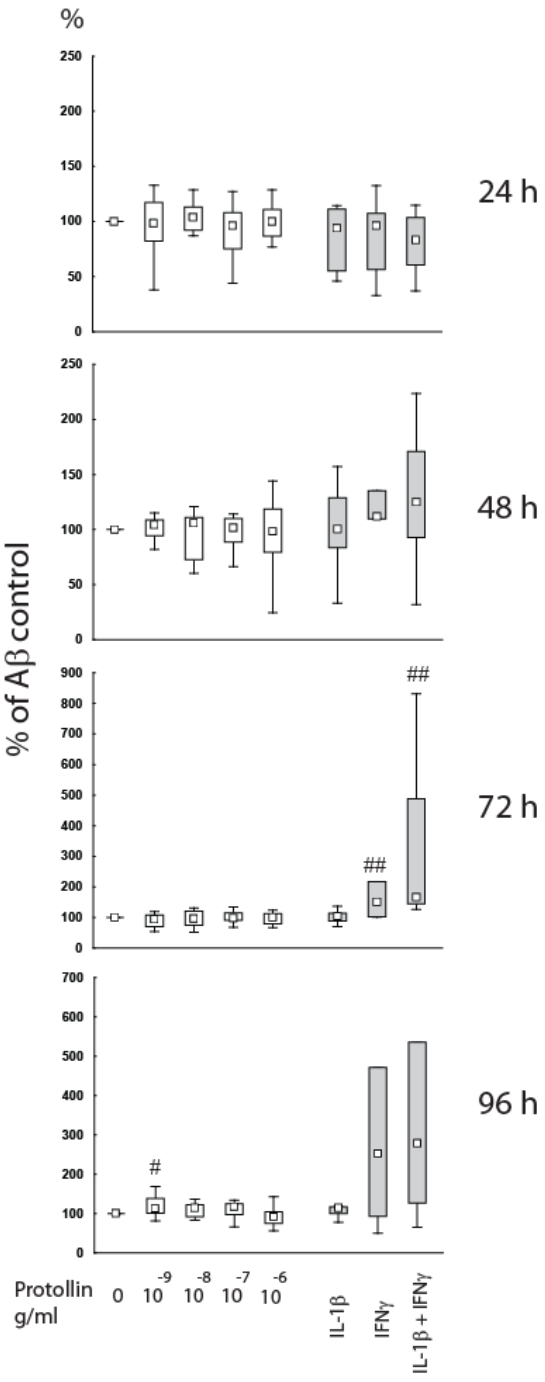

Fig. 4
Hjorth et al, 2009

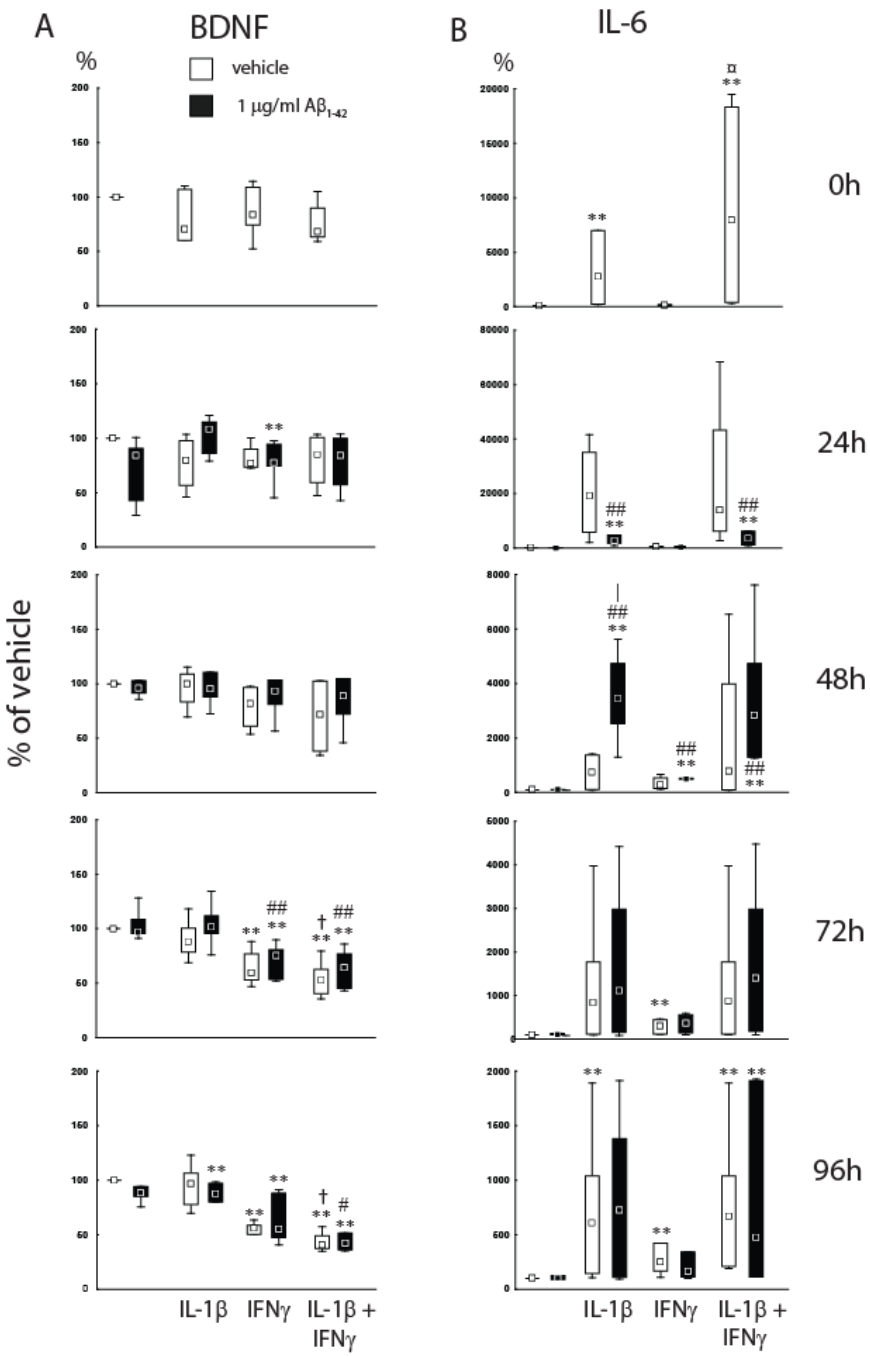

Fig. 5 A - B
Hjorth et al, 2009

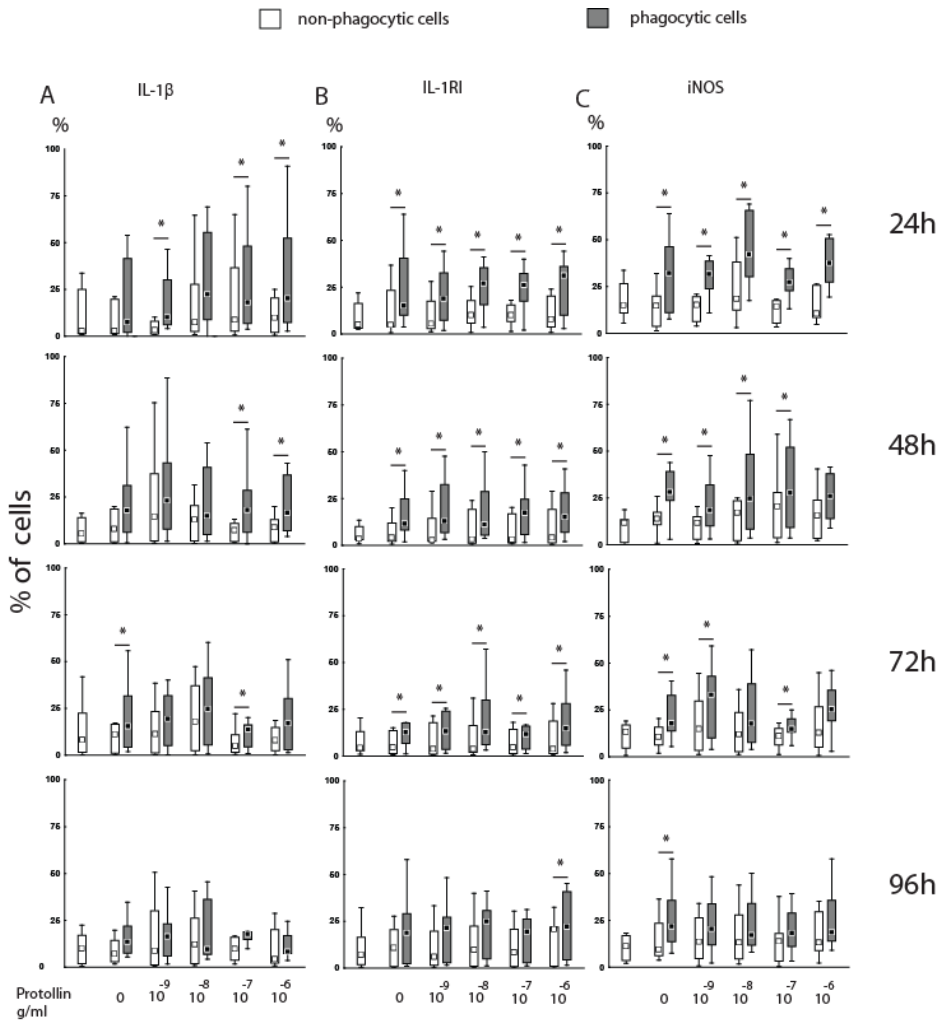

Fig. 6 A - C
Hjorth et al, 2009

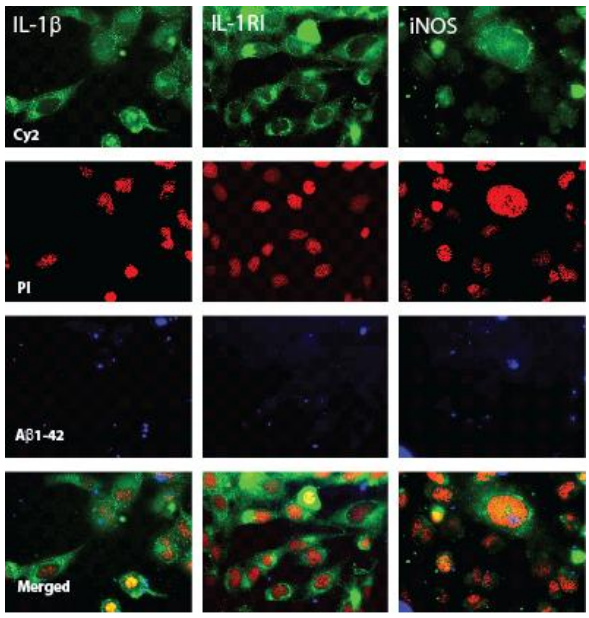

Fig. 7
Hjorth et al, 2009

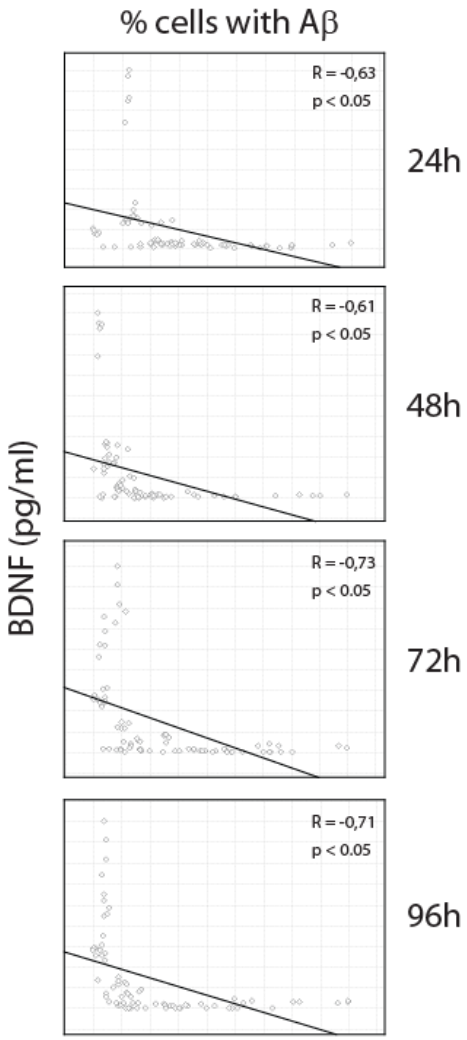

Fig. 8
Hjorth et al, 2009

III

Original Research Article

Dementia and Geriatric Cognitive Disorders

Dement Geriatr Cogn Disord 2009;27:481–490
DOI: 10.1159/000218081

Accepted: February 23, 2009
Published online: May 12, 2009

Effects of Omega-3 Fatty Acids on Inflammatory Markers in Cerebrospinal Fluid and Plasma in Alzheimer's Disease: The OmegAD Study

Yvonne Freund-Levi[a] Erik Hjorth[b] Catharina Lindberg[b] Tommy Cederholm[e] Gerd Faxen-Irving[c] Inger Vedin[d] Jan Palmblad[d] Lars-Olof Wahlund[a] Marianne Schultzberg[b] Hans Basun[f] Maria Eriksdotter Jönhagen[a]

Divisions of [a]Clinical Geriatrics, [b]Neurodegeneration and [c]Clinical Nutrition, Department of Neurobiology, Care Sciences and Society (NVS), and [d]Division of Haematology, Department of Medicine, Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, and [e]Clinical Nutrition and Metabolism and [f]Geriatrics, Department of Public Health and Caring Sciences, Uppsala University, Uppsala, Sweden

Key Words
Alzheimer's disease · Inflammation · Cerebrospinal fluid · ω-3 fatty acids · Cytokines

Abstract
Background: ω-3 fatty acids (ω-3 FAs) found in dietary fish or fish oils are anti-inflammatory agents that may influence Alzheimer's disease (AD). ***Objective:*** To study the effects of dietary ω-3 FA supplementation on inflammatory markers in cerebrospinal fluid (CSF) and plasma from patients with mild to moderate AD. ***Methods:*** Thirty-five patients (70.3 ± 8.2 years) were randomized to a daily intake of 2.3 g ω-3 FAs or placebo for 6 months. The inflammatory markers interleukin (IL)-6, tumour necrosis factor-α and soluble interleukin-1 receptor type II (sIL-1RII) were analysed in CSF and plasma at baseline and at 6 months. The AD markers tau-protein, hyperphosphorylated tau-protein and β-amyloid ($A\beta_{1-42}$) were assessed in CSF. High-sensitivity C-reactive protein was assessed in plasma. A possible relation to the *APOE* genotype was investigated. ***Results:*** There was no significant treatment effect of ω-3 FAs on inflammatory and AD biomarkers in CSF or on inflammatory markers in plasma, nor was there any relation with *APOE*. A significant correlation was observed at baseline between sIL-1RII and $A\beta_{1-42}$ levels in CSF. ***Conclusions:*** Treatment of AD patients with ω-3 FAs for 6 months did not influence inflammatory or biomarkers in CSF or plasma. The correlation between sIL-1RII and $A\beta_{1-42}$ may reflect the reciprocal interactions between IL-1 and Aβ peptides.

ClinicalTrials.gov. Identifier: NCT00211159

Introduction

Abundant evidence indicates that inflammatory processes are active in Alzheimer's disease (AD). Epidemiological surveys suggest a lower prevalence of AD in subjects treated with non-steroidal anti-inflammatory drugs (NSAIDs), but clinical trials have yielded limited effects [1, 2]. A central event in AD is the activation of microglia by different factors, including β-amyloid (Aβ) and pro-inflammatory cytokines [3, 4]. Microglia release cytokines, e.g. interleukin (IL)-1β, IL-6, and tumour necrosis factor-α (TNF-α), which may lead to neuronal dysfunction [5, 6]. Previous studies of inflammatory biomarkers in peripheral blood of AD patients have yielded inconclusive results. Serum as well as cerebrospinal fluid (CSF)

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1420–8008/09/0275–0481$26.00/0

Accessible online at:
www.karger.com/dem

Yvonne Freund-Levi, MD, PhD
Department NVS, Division of Clinical Geriatrics
Novum, 5th floor, Karolinska University Hospital, Huddinge
SE–141 86 Stockholm (Sweden)
Tel. +46 736 841 130, E-Mail Yvonne.Freund-Levi@ki.se

levels of soluble IL-6 receptor and TNF-α have been reported to be decreased, unchanged or increased [7, 8]. In patients with elevated TNF-α levels, cytokine-inactivating therapies are ongoing [9].

Neuronal cell membranes and synapses are enriched with polyunsaturated fatty acids such as the ω-3 fatty acid (ω-3 FA) docosahexaenoic acid (DHA). Synapse loss correlates with cognitive decline in AD, and synaptic function is affected by Aβ [10]. Fatty fish is the major dietary source of DHA. Epidemiological data imply that increased intake of fish or DHA can reduce the risk of developing AD [11, 12]. Animal data are also in line with these findings [13]. Interestingly, the DHA levels in serum and brain have been found to be lower in AD patients than in controls [14, 15]. DHA and eicosapentaenoic acid (EPA) are FAs found in the brain, incorporated as structural components of neural membrane glycerophospholipids, and are substrates for generating lipid mediators. The incorporation and proportions of ω-3 and ω-6 FAs markedly influence neural membrane properties such as fluidity and permeability, and gene expression. Oxygenation of ω-6 FAs generates pro-inflammatory mediators whereas actions of 15-lipoxygenase-like enzymes on DHA generate anti-inflammatory mediators.

These second messengers, the docosanoids, are potent endogenous anti-inflammatory and pro-resolving chemical lipid mediators [16]. Thus, growing evidence suggests that the generation of ω-3 FA metabolites may be an internal anti-inflammatory protective mechanism for preventing brain damage in neurodegenerative diseases [17–20]. DHA and EPA reduce chronic inflammation by attenuating NF-κB, in turn modulating the expression of pro-inflammatory cytokines including TNF-α and IL-1α and β. The intake of DHA and EPA reduces the synthesis of eicosanoids. How DHA and EPA decrease the activation of NF-κB is not clear at present. However, these FAs may decrease the phosphorylation of IκB, thereby modulating the availability of NF-κB. This process can modulate the expression of the pro-inflammatory genes for COX-2, intracellular adhesion molecule-1, vascular adhesion molecule-1, E-selectin, TNF-α, IL-β, IL-6, nitric oxide synthetase, and matrix metalloproteinases [21]. Collective evidence suggests that ω-3 FAs are associated with cognitive development, memory-related learning, neural membrane plasticity, synaptogenesis, and synaptic transmission, and suppress the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, and IL-6 [20].

This provides us with an opportunity to use DHA as a nutraceutical or pharmaceutical tool in brain disorders such as AD [22]. The majority of the published epidemiological studies are consistent with a positive association between reported high DHA consumption or high DHA blood levels and a lower risk of developing AD later in life [15]. Such observations have prompted analysis of the effects of DHA in transgenic animal models of AD, showing that DHA exerts a beneficial effect against Aβ accumulation, cognitive impairment, synaptic marker loss, and hyperphosphorylation of tau [13]. Multiple mechanisms of action can be associated with the neuroprotective effects of DHA and include antioxidant properties and activation of distinct cell-signalling pathways [18]. Inflammation is a component of a range of acute and chronic human diseases, and is characterized by the production of inflammatory cytokines, arachidonic-acid-derived eicosanoids, other inflammatory mediators (e.g. platelet-activating factor) and adhesion molecules. ω-3 polyunsaturated FAs decrease the production of inflammatory mediators and the expression of adhesion molecules. They act both directly (e.g. by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism) and indirectly (e.g. by altering the expression of inflammatory genes through effects on transcription factor activation). Thus, ω-3 FAs could be potent anti-inflammatory agents. As such, they have been proposed as therapeutic agents for AD [22, 23].

In a recently performed randomized placebo-controlled trial, the OmegAD Trial, 174 patients with mild to moderate AD received either treatment with the ω-3 FA preparation or placebo for 6 months, followed by 6 months with ω-3 FA treatment in both groups. The trial was power calculated to detect statistical differences on cognition using the cognitive portion of the Alzheimer Disease Assessment Scale (ADAS-Cog) [24] and the Mini Mental Test Examination (MMSE) [25]. Administration of 2.3 g ω-3 FAs (1.7 g DHA and 0.6 g EPA) did not delay the rate of cognitive decline according to the MMSE or the ADAS-Cog [26], nor was there any effect on neuropsychiatric symptoms [27]. However, in a small group (n = 32) of patients with very mild AD (MMSE >27), the cognitive decline was postponed [26].

In the present report of the OmegAD Trial, we have studied whether supplementation of AD patients with ω-3 FAs affects the levels of inflammatory markers in CSF and plasma. The possible relation to *APOE* genotype was also investigated. Since high concentrations of high-sensitivity C-reactive protein (hs-CRP) have been proposed as a predictor of cognitive decline and dementia [28], we also analysed the plasma levels of this inflammatory marker.

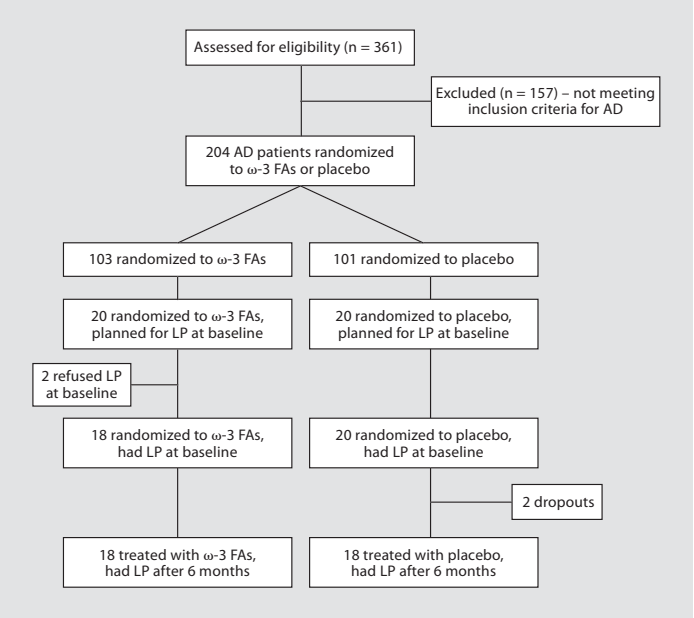

Fig. 1. Patient flow chart.

Material and Methods

Subjects and Study Design

This study was part of a larger randomized, double-blind placebo-controlled trial conducted between December 13, 2000, and March 25, 2004, in which 204 patients were enrolled [26, 27]. The Ethical Committee at Karolinska University Hospital approved the study protocol. Before entering the study, both the patients and knowledgeable informants (most often caregiver) signed an informed consent. The inclusion criteria required that the patients had a diagnosis of AD according to the DSM-IV criteria [29], had an MMSE [25] score between 15 and 30, were living in their own home, and had been treated with a stable dose of acetylcholine esterase inhibitors (AChEIs) for at least 3 months before the study start. Patients were excluded if treated with NSAIDs (low-dose acetylsalicylic acid was accepted), ω-3 FA preparations, anticoagulation agents, or if they abused alcohol, suffered from a concomitant serious disease, or did not have a caregiver. The patients were randomized in blocks of four in sealed envelopes to receive four 1-gram capsules daily, each containing either 430 mg DHA (22:6 ω-3 FA) and 150 mg EPA (20:5 ω-3 FA), i.e. EPAX 1050TG (Pronova Biocare A/S, Lysaker, Norway), or an isocaloric placebo oil (containing 1 g of corn oil, including 0.6 g of linoleic acid, here denoted placebo) for 6 months. EPAX1050TG is a 60% ω-3 FA concentrate in triglyceride form produced according to Good Manufacturing Practice. Four milligrams of vitamin E (tocoferol) was added to each capsule. The reason for choosing a DHA-enriched compound was that a significant reduction in total Aβ by 70% compared to control chow diets was found in animal studies [13], and that patients with AD have lower levels of DHA in their brains [14].

Patients in the OmegAD Trial underwent the following evaluations: routine blood and urine sampling, assessment of blood pressure, body mass index (BMI), and cognitive function using the MMSE and the modified ADAS-Cog [24], neuropsychiatric evaluation using the Neuropsychiatric Inventory (NPI) [30] and evaluation of depression using the Montgomery and Asberg Depression Rating Scale [31]. Due to ethical considerations, approval for 40 patients to undergo lumbar puncture (LP) was given by the ethical committee. According to the study protocol, the first consecutive 40 patients enrolled in the study were asked to undergo LP at baseline and after 6 months. Two patients (from the ω-3 FA group) did not want to perform LP and 2 patients (from the placebo group) dropped out within the first 12 weeks due to stomach pain and headache (fig. 1). Data from 1 patient were excluded due to extreme values and thus the final analysis was based on 35 patients.

Blood samples for analysis of serum FA levels were obtained to assess compliance with the ω-3 FA therapy. The data on ω-3 FA levels all included 204 patients and were presented by Freund-Levi et al. [26].

The primary efficacy variables for the OmegAD Trial were cognitive functions assessed by MMSE and ADAS-Cog. Secondary outcomes were safety and tolerability assessed by the Clinical Dementia Rating Scale global and summary of boxes [26].

This paper is based on CSF and plasma data from 35 patients at baseline and after 6 months treatment with ω-3 FAs or placebo (fig. 1).

Sample Collection and Analysis of CSF and Plasma

Blood, plasma and CSF samples from the 35 patients were collected at baseline and at 6 months. The LP was performed in a standardized manner with all patients in a sitting position between 11 a.m. and 1 p.m.; the tap was performed with a non-traumatic cannula placed in the intervertebral space L3/L4 or L4/L5. Five millilitres of CSF were collected in sterile polypropylene tubes and put on ice and centrifuged according to standard routine at 3,000 rpm for 10 min at 4°C. The supernatants were aliquoted and stored at –70°C until analysis.

Plasma samples were obtained by venous puncture and collected in 1.25-ml Na-heparin tubes and centrifuged according to the standard procedure at 3,000 rpm for 10 min at 4°C and the supernatants were aliquoted and kept at –70°C until analysis.

The levels of IL-6, TNF-α, and soluble interleukin-1 receptor type II (sIL-1RII) were analysed in CSF and plasma samples by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Abingdon, UK). The detection range was 0.16–10 pg/ml for IL-6 and 31.3–2,000 pg/ml for sIL-1RII in both plasma and CSF. For TNF-α, the lowest detectable range was 0.038–0.191 pg/ml in CSF samples and 15.6–1,000 pg/ml in plasma samples. The levels of hs-CRP in plasma were analysed using a commercially available kit from LX 20 (Beckman AB, Bromma, Sweden) and performed at the Department of Clinical Chemistry, Karolinska University Hospital. A near-infrared immunoassay rate method based on the binding of an anti-CRP antibody-coated particle that binds to CRP in the presence of a patient sample was used. The detection range was 0.2–380 mg/l.

Frozen CSF samples were sent on dry ice to the Clinical Neurochemistry Laboratory, Mölndals Lasarett, Gothenburg, Sweden, for analysis of $A\beta_{1-42}$, and tau protein (T-tau). The total levels of CSF T-tau were determined using a sandwich ELISA constructed to detect both normal and hyperphosphorylated tau (P-tau) [32]. CSF P-tau levels were determined using a sandwich ELISA, constructed to specifically detect tau phosphorylated at Thr181 [33]. The levels of $A\beta_{1-42}$ were analyzed in CSF samples using a sandwich ELISA, constructed to specifically measure $A\beta_{1-42}$ [34].

Serum samples for assessment of serum FA levels were analysed by gas chromatography (TR-Fame column, 30 m × 0.32 mm ID × 0.25 μm film gas chromatography column; Thermo Electron Corp., Waltham, Mass., USA). For details of the analysis and the full results, see Freund-Levi et al. [26] and Boberg et al. [35].

Patient DNA samples for *APOE* genotype analysis were extracted from peripheral white blood cells using standard methods [36] and the *APOE* genotype was determined by a microsequencing method on microtiter plates (AffiGene ApoE; Sangtec Medical, Bromma, Sweden).

Statistical Analysis

Data are presented as mean and 95% confidence interval (CI) or standard deviation (SD). Between- and within-group analyses of skewed variables were performed by the Mann-Whitney U test. Due to missing data at baseline or after 6 months, the effects of ω-3 FA and time on the levels of IL-6, sIL-1RII, TNF-α, P-tau, T-tau and $A\beta_{1-42,}$ were analysed using linear mixed-effect models.

Table 1. Demographic data at baseline

	ω-3 FAs (n = 18)	Placebo (n = 17)	p value
Age, years	72.2 ± 8.8	68.3 ± 7.3	0.1
Females, n	8 (44%)	6 (30%)	1.0
APOEε4, n			
0	4 (22%)	2 (12%)	
1	9 (50%)	9 (53%)	
2	5 (28%)	6 (35%)	
Acetyl salicylic acid, n	4 (22%)	2 (10%)	0.3
Systolic blood pressure, mm Hg	143 ± 20	134 ± 10	0.9
Diastolic blood pressure, mm Hg	78 ± 6	77 ± 6	0.3
BMI	25.9 ± 2.2	23.7 ± 2.9	0.03[1]
MMSE score	24.3 ± 3.8	23.9 ± 4.0	0.8
ADAS-Cog score	22.8 ± 10.9	29.2 ± 7.1	0.02[1]
MADRS score	1.7 ± 2.3	2.2 ± 3.2	0.9
NPI score	12.3 ± 8.5	17.7 ± 14.3	0.3

MMSE, 0–30 points; ADAS-Cog, 0–85 points; MADRS = Montgomery Åsberg Depression Rating Scale, 0–30 points; NPI, 0–144 points.

[1] Significance of differences between groups using the Mann-Whitney U test.

In all models, gender, age, *APOE*, NPI, ADAS-Cog, BMI, treatment group, time and the interaction between treatment and time were controlled for. The outcome variables, IL-6, sIL-1RII, TNF-α, and T-tau, were positively skewed, or expressed heterogeneous variance and therefore a logarithmic scale was used. In table 2, the results from the analysis of the skewed variables have been back-transformed. In this case, the data are expressed in relative values rather than absolute values; hence the results from these analyses are presented in percentages (i.e. the ratio between ω-3 FA treatment and placebo at 6 months).

A backward selection was performed to evaluate which variables to include in the final models. An exclusion criterion of $p > 0.05$ was used. Cook's distance was used to reveal observations with a substantial influence on the results. One such observation was found in the analysis of IL-6, P-tau, T-tau and $A\beta_{1-42}$, and was excluded from the analysis.

The statistical analysis was carried out with SPSS (SPSS for Windows, Rel. 16.02; SPSS Inc., Chicago, Ill., USA) or the Statistica® 7.0 software package (Stat soft, Tulsa, Okla., USA).

Results

Demographic, clinical and cognitive data from the 35 patients are shown in table 1. There was a significant difference at baseline between the ω-3 FA and the placebo groups in the ADAS-Cog scores ($p = 0.02$) and BMI ($p = 0.03$). After 6 months of treatment, these differences be-

Table 2. Parameter estimates of log-and non-transformed outcome variables using linear mixed effect models. In all models, gender, age, *APOE*, NPI, ADAS-cog, BMI, treatment group, time and the interaction between treatment and time were controlled for

Comparison	Estimate: ratio[1] or difference[2]	SE	p value	95% CI	
				lower limit	upper limit
IL-6 plasma[1]					
ω-3 FA: time 6 vs. 0	26%	NA	0.07	–3%	46%
Placebo: time 6 vs. 0	30%	NA	0.03	5%	49%
Time 0: ω-3 vs. placebo	21%	NA	0.34	–18%	68%
Time 6: ω-3 vs. placebo	13%	NA	0.54	–24%	31%
IL-6 CSF[1]					
ω-3 FA: time 6 vs. 0	–2	NA	0.76	–13	9
Placebo: time 6 vs. 0	–11	NA	0.06	–24	0
Time 0: ω-3 vs. placebo	11	NA	0.37	–13	41
Time 6: ω-3 vs. placebo	22	NA	0.1	–4	54
sIL-1RII CSF[1]					
ω-3 FA: time 6 vs. 0	–2	NA	0.79	–15	11
Placebo: time 6 vs. 0	–4	NA	0.53	–11	13
Time 0: ω-3 vs. placebo	–16	NA	0.17	–35	9
Time 6: ω-3 vs. placebo	–14	NA	0.23	–33	49
sIL-1RII plasma[1]					
ω-3 FA: time 6 vs. 0	3	NA	0.77	–20	22
Placebo: time 6 vs. 0	–3	NA	0.79	–26	16
Time 0: ω-3 vs. placebo	–4	NA	0.76	–15	20
Time 6: ω-3 vs. placebo	2	NA	0.85	–20	20
TNF-α CSF[1]					
Time 0: Placebo vs. ω-3	–1	NA	0.97	–53	106
Time 6: Placebo vs. ω-3	–28	NA	0.41	–38	209
ω-3 FA: time 6 vs. 0	–89	NA	< 0.001	–94.8	–77
Placebo: time 6 vs. 0	–85	NA	< 0.001	–93.1	–67
TNF-α Plasma[1]					
Time 0: Placebo vs. ω-3	–17	NA	0.85	–88	495
Time 6: Placebo vs. ω-3	27	NA	0.81	–82	805
ω-3 FA: time 6 vs. 0	14	NA	0.54	–43	35
Placebo: time 6 vs. 0	73	NA	0.02	8	179
T-tau CSF[1]					
ω-3 FA: time 6 vs. 0	–2.4	NA	0.37	–8.1	3
Placebo: time 6 vs. 0	–5	NA	0.05	–11	1
Time 0: ω-3 vs. placebo	0	NA	0.99	–27	37
Time 6: ω-3 vs. placebo	3	NA	0.85	–25	40
APOE 0 vs. 1-2	67	NA	0.02	10	154
P-tau CSF[2]					
ω-3 FA: time 6 vs. 0	–5.85	3.20	0.08	–12.39	0.70
Placebo: time 6 vs. 0	–7.28	3.06	0.02	–13.52	1.05
Time 0: ω-3 vs. placebo	–1.31	8.40	0.88	–18.3	15.68
Time 6: ω-3 vs. placebo	0.13	8.31	0.99	–16.70	16.95
APOE 0 vs. 1-2	27.73	11.13	0.02	5.09	50.37
$A\beta_{1-42}$ CSF[2]					
ω-3 FA: time 6 vs. 0	–3.33	14.55	0.82	–41.91	35.24
Placebo: time 6 vs. 0	–2.02	15.29	0.9	–42.45	38.41
Time 0: ω-3 vs. placebo	–37.39	32.40	0.26	–121.77	47.00
Time 6: ω-3 vs. placebo	–36.07	32.06	0.27	–119.68	47.54

[1] Ratio between means expressed as percentage. log-transformed outcome variable. [2] Mean difference between groups. Non-transformed outcome variable. Time 0 = At baseline; time 6 = at 6 months.

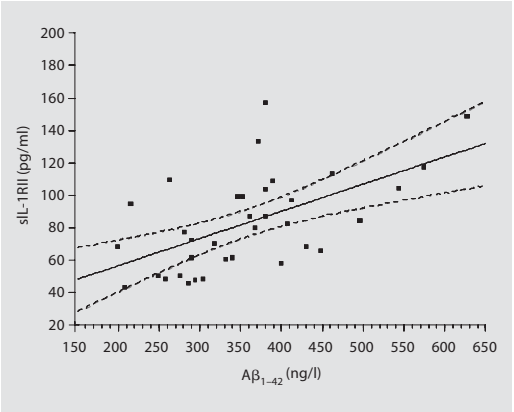

Fig. 2. Positive linear relationship between sIL-1RII and $A\beta_{1-42}$ in CSF at baseline in AD patients (r = 0.56, p = 0.001, n = 33).

tween the groups did not remain (p = 0.3 for ADAS-Cog scores and p = 0.2 for BMI, data not shown). There was no significant interaction between age, gender, *APOE*, MMSE, NPI, ADAS-Cog, treatment or levels of IL-6, TNF-α, sIL-1RII or $A\beta_{1-42}$ over time (0–6 months) (table 2). The plasma levels of EPA, DHA and linoleic oil at baseline or 6 months did not affect the levels of IL-6, TNF-α, sIL-1RII, $A\beta_{1-42}$, T-tau or P-tau (data not presented).

Inflammatory and Dementia Biomarkers in CSF

IL-6, TNF-α, and sIL-1RII. The levels of sIL-1RII were positively correlated to $A\beta_{1-42}$ levels at baseline (r = 0.56; p = 0.001, fig. 2) but not after 6 months of treatment. There was no significant difference at baseline or at 6 months between the ω-3 FA and the placebo group with regard to CSF levels of IL-6, TNF-α, or sIL-1RII (table 3), nor was there any significant effect of treatment over time between groups (table 3), or within each group, except for levels of TNF-α, which significantly decreased in both groups over time ($p < 0.001$ within each group).The levels of IL-6 were (8%) higher in the ω-3 FA group than in the placebo group at the 6-month time point although the differences did not reach statistical significance.

$A\beta_{1-42}$, T-tau and P-tau. Strong correlations were found between the levels of T-tau and P-tau at baseline (r = 0.95, $p < 0.001$) and at 6 months (r = 0.96, $p < 0.001$). There was no significant difference in $A\beta_{1-42}$, T-tau and

Table 3. Analysis of inflammatory and dementia biomarkers in CSF in ω-3 FA and placebo groups at baseline and after 6 months follow-up[1]

Variables	Baseline		6 months		0–6 months
	mean level	p value	mean level	p value	p value
IL-6, pg/ml					
ω-3 FAs	3.2 (2.6–3.8)	1.0	3.3 (2.7–4.0)	0.3	0.3
Placebo	3.7 (2.3–5.0)		2.7 (2.3–3.1)		
sIL-1RII, pg/ml					
ω-3 FAs	80.2 (65.6–94.9)	0.6	79.6 (64.5–94.7)	1.0	1.0
Placebo	85.6 (70.1–101.2)		84.7 (63.0–106.3)		
TNF-α, pg/ml					
ω-3 FAs	0.98 (0.7–1.2)	1.0	0.25 (0.1–0.4)	0.9	0.7
Placebo	0.96 (0.8–1.2)		0.18 (0.1–0.3)		
$A\beta_{1-42}$, ng/l					
ω-3 FAs	345.7 (289.1–402.3)	0.4	349.0 (307.8–390.2)	0.4	0.8
Placebo	371.1 (327.1–415.1)		365.3 (325.6–405.1)		
T-tau, ng/l					
ω-3 FAs	596.2 (454.1–738.3)	0.5	597.0 (460.6–733.5)	0.9	0.8
Placebo	720.8 (540.9–900.8)		616.9 (532.7–701.1)		
P-tau, ng/l					
ω-3 FAs	79.1 (63.2–95.0)	0.4	76.9 (62.5–91.3)	0.9	0.9
Placebo	91.5 (72.4–110.5)		78.3 (68.0–88.7)		

95% CIs are shown in parentheses.
[1] Significance of difference between the ω-3 FA and placebo groups and change over time using the Mann-Whitney U-test.

Table 4. Analysis of plasma biomarkers in ω-3 FA and placebo groups at baseline and after 6 months follow-up[1]

Variables	Baseline		6 months		0–6 months
	mean level	p value	mean level	p value	p value
IL-6, pg/ml					
ω-3 FAs	1.4 (0.7–2.0)	0.2	1.5 (1.0–1.9)	0.3	0.8
Placebo	0.7 (0.5–1.0)		1.1 (0.7–1.5)		
sIL-1RII, pg/ml					
ω-3 FAs	1,591 (1,264–1,917)	0.8	1,771 (1,381–2,163)	0.8	0.3
Placebo	1,624 (1,287–1,961)		1,556 (1,291–1,821)		
TNF-α, pg/ml					
ω-3 FAs	105.8 (–4.0 to 216)	0.6	110 (–19 to 238)	0.6	1.0
Placebo	103.3 (15.5–191.0)		104.0 (23.5–184.6)		
hs-CRP, mg/l					
ω-3 FAs	1.5 (0.5–2.5)	0.6	1.7(0.5–2.9)	0.4	0.4
Placebo	0.9 (0.5–1.2)		1.5 (0.2–2.7)		

95% CIs are shown in parentheses.
[1] Significance of difference between the ω-3 FA and placebo groups and change over time using the Mann-Whitney U-test.

P-tau levels between the ω-3 FA and the placebo group at baseline or at 6 months, nor was there any effect of treatment over time (table 3). The T-tau levels at baseline were almost twice as high in *APOEε4* carriers (711.1 pg/ml, n = 29) as compared to non-*APOEε4* carriers (394.2 pg/ml, n = 6, p = 0.03). At baseline, there were also significantly higher P-tau levels in *APOEε4* carriers (91.3 pg/ml, n = 29) as compared to non-*APOEε4* carriers (55.3 pg/ml, n = 6, p = 0.03). As there were only 2 non-*APOEε4* carriers in the placebo group, it was not possible to evaluate interaction with ω-3 FA and placebo treatment.

Inflammatory Biomarkers in Plasma

Plasma levels of IL-6, TNF-α, sIL-1RII and hs-CRP did not differ significantly either at baseline or after 6 months between the ω-3 FA and placebo group, nor was there any significant effect of treatment over time (table 4).

Discussion

In the present study, we have analysed CSF and plasma levels of IL-6, TNF-α and sIL-1RII as well as the levels of T-tau, P-tau and $A\beta_{1-42}$ in CSF in patients with mild to moderate AD, upon treatment with ω-3 FAs for 6 months. In addition, the possible relation to *APOE* genotype was investigated since *APOEε4* is a major risk factor for developing AD [37]. Treatment with ω-3 FAs resulted in null effects on the inflammatory markers in CSF and in plasma. Nor was there any effect of treatment on the levels of dementia biomarkers as compared to placebo. In cell cultures and animal studies as well as in epidemiological surveys, agents known to decrease inflammation, such as vitamins, anti-oxidants, *Gingko biloba* and long-term NSAIDs, have shown protective effects against AD pathology or reduced risk for dementia [38, 39]. Randomized controlled clinical trials using these anti-inflammatory agents have, however, failed to show clear positive treatment benefits [1]. Some of the difficulties to perform clinical trials on whether anti-inflammatory drugs can delay or prevent adverse health events in the field of AD can be due to the length of time needed to demonstrate a difference with treatment. This has been discussed using the Alzheimer's Disease Anti-inflammatory Prevention Trial as an example. Similarly to other treatment trials, those targeting AD dementia balance these benefits against the risks of treatment. In contrast, the benefit seen in prevention trials, if any, will be found only in the absence or delay in disease onset, often after years of continuous treatment. Other difficulties relate to the length of prevention trials and dosages [2].

Low intake of ω-3 FAs has been found to be a risk factor for developing dementia [12] and dietary DHA has been shown to reduce amyloid burden in transgenic mice [13]. From animal studies it is also known that ω-3 FAs exert anti-inflammatory actions such as decreasing arachidonic-acid-derived eicosanoids with pro-inflammatory properties, increasing EPA-derived eicosanoids with less pro-inflammatory effects, and increasing the generation of EPA- and DHA-derived anti-inflammatory resolvins. In a mouse model of rheumatoid arthritis, the levels of the inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 were reduced in response to ω-3 FA treatment [40].

Some studies on cytokine levels in clinical samples have shown elevated CSF levels of sIL-1RII in mild to moderate AD [41], and elevated levels of TNF-α in CSF and plasma of AD patients [42, 43]. The CSF levels of sIL-1RII and IL-6 in the present study confirm our earlier findings [41] and those of Jia et al. [42], respectively. The range of plasma levels of IL-6 are similar to those found by Zuliani et al. [43]. Recently, plasma levels of IL-1 and TNF-α were indicated as strong predictors for development of AD [44].

The present data from the OmegAD study did not show any effects of ω-3 FA treatment on inflammatory markers in CSF (sIL-1RII, IL-6, TNF-α) and plasma (sIL-1RII, IL-6, TNF-α, hs-CRP). This may be explained by the limited number of CSF samples or that there is no drug effect. The results can also be due to a factual null effect. Similarly, the pretreatment differences concerning the scores of ADAS-Cog and BMI can be based on the low number of patients selected for LP. Furthermore, the treatment with ω-3 FA could affect other biomarkers than the ones analysed in the present study. However, our data are consistent with the lack of effect on cognition and neuropsychiatric symptoms previously reported in these patients although some positive effects were found in subsets of patients [26, 27]. Thus, the ω-3 FA treatment was found to postpone cognitive decline in patients with very mild AD [26]. Whether or not the inflammatory markers were affected in this subgroup of patients awaits further studies, since only very few of these patients were among those selected for LP.

TNF-α levels in CSF were significantly reduced in both groups over time, suggesting a methodological influence rather than a biological effect.

The significant difference between ADAS-Cog scores as well as BMI of the two groups at baseline is cumbersome since they were strictly randomized in blocks of

four. In the OmegADtrial on 174 patients previously reported, the treatment and the placebo groups did no differ at baseline [26]. The explanation for this discrepancy may be that the significances at baseline reported in this paper are due to a chance significance because of the smaller sample number.

All patients in the present study were on standard treatment with AChEIs, therefore representing a relevant clinical sample of patients with AD. AChEIs have been linked to anti-inflammatory properties both in animal and human studies, and were shown to reduce the levels of cytokines such as IL-6 [45, 46]. In view of this, the concomitant treatment with AChEIs in the present study may have masked a smaller anti-inflammatory effect of the ω-3 FAs, and may be one of several explanations for the lack of effect on the inflammatory markers observed in our AD patients.

Another explanation may be the dose of ω-3 FAs (2.3 g; 1.7 g DHA + 0.6 g EPA/day) used: a higher dose may be needed to attenuate the inflammation. To our knowledge, there are two short-term clinical trials of ω-3 FAs in AD patients using doses of 240 mg/day of DHA and arachidonic acid [47] or 0.25 ml α-linoleic acid [48], and showing improvement in cognition [47] and quality of life [48], but these studies did not investigate inflammatory markers. Other studies with DHA in the field of AD are ongoing [49].

The proportion of DHA and EPA in the ω-3 FA supplementation may be of significance since EPA and DHA display partly different modes of action. Thus, DHA binds to the RXR receptor, enhances membrane fluidity and exerts neuroprotection [17, 21]. Furthermore, the brain is rich in DHA, but contains virtually no EPA, suggesting that a higher proportion of DHA as compared to EPA may be beneficial in trials on AD patients, even if EPA and DHA to some extent can be metabolized to each other. Although many studies have studied the effects of mainly EPA-rich fish oils on inflammatory reactions, few have investigated the effects of mainly DHA-rich fish oils. Effects of ω-3 FA treatment on the levels of $A\beta_{1-42}$, T-tau and P-tau in CSF from AD patients have so far not been studied. A 6-month ω-3 FA treatment with the doses used here did not affect these dementia biomarkers. The stability of the biomarkers over time is not fully clear although several studies have shown unchanged levels for at least 1 year in patients with AD [50] while in patients with mild cognitive impairment who convert to AD, P-tau has been found to increase over time [51]. Since the treatment duration here was only 6 months, the AD biomarkers in the placebo group did not, as expected, change.

Analyses of the inflammatory markers and dementia biomarkers in relation to the *APOE* genotype did not reveal a significant association, but the treatment and placebo groups as well as the number of non-*APOEε4* carriers may have been too small to detect such correlations.

Correlation analysis between inflammatory markers and the AD biomarkers showed a positive correlation at baseline between sIL-1RII and $A\beta_{1-42}$ in the CSF. A related finding was seen in transgenic mice, in which a strong correlation was observed between the brain levels of $A\beta_{1-42}$ and the cytokines IL-1α and IL-1β [52]. Such a correlation was also seen with regard to the levels of IL-1β, IL-6 and TNF-α in normal mouse brains [52]. The levels of sIL-1RII in CSF were shown to be elevated in a group of patients with mild to moderate AD [41], whereas no significant difference was seen in patients with mild cognitive impairment, nor at later stages of the disease [53]. Since sIL-1RII binds to IL-1β and acts as a 'sink' for IL-1β, the increase in sIL-1RII shedding may be an attempt to limit negative effects of IL-1β expression and activity in the brain [54]. Interestingly, after 6 months of treatment with ω-3 FAs or placebo, the sIL-1RII and $A\beta_{1-42}$ correlation found at baseline had disappeared. However, a higher number of patients in each group may be required to see a differential effect between ω-3 FAs and placebo. Further research is needed to clarify the possible link between sIL-1RII and amyloid in CSF in AD.

For future research, long-term randomized placebo-controlled studies of different dosages of DHA in patients with mild cognitive impairment and pre-AD including analyses of inflammatory markers in CSF and plasma are warranted.

Acknowledgements

Financial support was provided through the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet, Funds of Capio, Demensförbundet, Stiftelsen Gamla Tjänarinnor, Swedish Alzheimer Foundation, Odd Fellow, Swedish Nutrition Foundation, Gun och Bertil Stohnes Stiftelse, Swedish Society of Physicians and Lion's Sweden. The OmegAD study was initially partly funded by Pronova Biocare A/S, Lysaker, Norway. The company was represented in the trial steering committee with regard to study design, and provided the EPAX1050TG and placebo preparations; the company was not involved in data and patient collection, analyses or interpretation of scientific data.

We thank A.-C. Tysen-Bäckström, RN, and Andreas Svensson, RN, for patient data and sample management, and Jakob Bergström, MSc, LIME, Karolinska Institutet, for statistical advice.

References

1 Tabet N, Feldman H: Ibuprofen for Alzheimer's disease Cochrane Database Syst. Rev 2003 Art No: CD004031.

2 Meinert CL, Breitner JC: Chronic disease long-term drug prevention trials: lessons from the Alzheimer's Disease Anti-Inflammatory Prevention Trial (ADAPT). Alzheimers Dement 2008;4:(suppl 1):S7–S14.

3 Engelhart MJ, Geerlings MI, Meijer J, Kiliaan A, Ruitenberg A, van Swieten JC, Stijnen T, Hofman A, Witteman JC, Breteler MM: Inflammatory proteins in plasma and the risk of dementia. The Rotterdam Study. Arch Neurol 2004;61:668–672.

4 Patel NS, Paris D, Mathura V, Quadros AN, Crawford FC, Mullan MJ: Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J Neuroinflammation 2005;2:1–10.

5 Farfara D, Lifschitz V, Frenkel D: Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. J Cell Med 2008;12:762–780.

6 Schultzberg M, Lindberg C, Aronsson Forslin Å, Hjorth E, Spulber SD, Oprica M: Inflammation in the nervous system-physiological and pathophysiological aspects. Physiol Behav 2007;92:121–128.

7 Mrak RE, Griffin WS: Potential Inflammatory biomarkers in Alzheimer's disease. J Alzheimers Dis 2005;8:369–375.

8 Tarkowski E, Blennow K, Wallin A, Tarkowski A: Intracerebral production of tumor necrosis factor-α, a local neuroprotective agent, in Alzheimer disease and vascular dementia. J Clin Immunol 1999;19:223–230.

9 Tobinick E, Gross H, Weinberger A, Cohen H: TNF-α modulation for treatment of Alzheimer's disease: 6-month pilot study. Med Gen Med 2006;8:25.

10 Scheff SW, Price DA, Schmitt FA, Mufson EJ: Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. Neurobiol Aging 2006;27:1372–1384.

11 Cole GM, Lim GP, Yang F, Teter B, Begum A, Ma Q, Harris-White ME, Frautchsy SA: Prevention of Alzheimer's disease: Omega 3 fatty acid and phenolic anti-oxidant interventions. Neurobiol Aging 2005;26 S1:133–136.

12 Kalmijn S, Launer LJ, Alewijn O, Witteman JC, Hofman A, Breteler MM: Dietary fat intake and the risk of incident dementia in the Rotterdam Study. Ann Neurol 1997;42:776–782.

13 Lim GP, Calon F, Morihara T, Yang F, Teter B, Ubeda O, Salem N Jr, Frautschy SA, Cole GM: A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. J Neurosci 2005;25:3032–3040.

14 Söderberg M, Edlund C, Kristensson K, Dallner G: Fatty acid composition of brain phospholipids in aging in Alzheimer's disease. Lipids 1991;26:421–425.

15 Conquer JA, Tierney MC, Zecevic J, Bettger WJ, Fisher RH: Fatty acid analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment. Lipids 2000;35:1305–1312.

16 Serhan CN: Mediator lipidomics. Prostaglandins Other Lipid Mediat 2005;77:4–14.

17 Mukherjee PK, Chawla A, Loayza MS, Bazan NG: Docosanoids are multifunctional regulators of neural cell integrity and fate: significance in aging and disease. Prostaglandins Leukot Essent Fatty Acids 2007;77:233–238.

18 Calon F, Cole G: Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies. Review. Prostaglandins Leukot Essent Fatty Acids 2007;77:287–293.

19 Lukiw WJ, Cui JG, Marcheselli VL, Bodker M, Botkjaer A, Gotlinger K, Serhan CN, Bazan NG: A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. J Clin Invest 2005;115:2774–2783.

20 Farooqui AA, Ong WY, Horrocks LA, Cheng P, Farooqui T: Comparison of biochemical effects of statins and fish oil in the brain: the battle of the titans. Brain Res Rev 2007;56:443–471.

21 Calderon F, Kim HY: Role of RXR in neurite outgrowth induced by docosahexaenoic acid. Prostaglandins Leukot Essent Fatty Acids 2007;77:227–232.

22 Issa AM, Mojica WA, Morton SC, Traina S, Newberry SJ, Hilton LG, Garland RH, MacLean CH: The efficacy of omega-3 fatty acids on cognitive functions in aging and dementia: a systematic review. Dement Geriatr Cogn Disord 2006;21:88–96.

23 Calder PC: Dietary modification of inflammation with lipids. Review. Proc Nutr Soc 2002;61:345–358.

24 Mohs RC, Knopman D, Petersen RC, Ferris SH, Ernesto C, Grundman M, Sano M, Bieliauskas L, Geldmacher D, Clark C, Thal LJ: Development of cognitive instruments for use in clinical trials of antidementia drugs; additions to the Alzheimer's Disease Assessment Scale that broaden its scope. The Alzheimer's Disease Cooperative Study. Alzheimer Dis Assoc Disord 1997;11(suppl 2):S13–S21.

25 Folstein MF, Folstein SE, McHugh PR: 'Mini-Mental State'. A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–198.

26 Freund-Levi Y, Eriksdotter Jönhagen M, Cederholm T, Basun H, Faxen-Irving G, Garlind A, Vedin I, Vessby B, Wahlund LO, Palmblad J: Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer's disease: OmegAD Study. Arch Neurol 2006;63:1402–1408.

27 Freund-Levi Y, Basun H, Cederholm T, Faxen-Irving G, Garlind A, Grut M, Vedin I, Palmblad J, Wahlund LO, Eriksdotter Jönhagen M: Omega-3 supplementation in mild to moderate Alzheimer's disease: effects on neuropsychiatric symptoms. Int J Geriatr Psychiatry 2008;23:161–169.

28 Kuo HK, Yen CJ, Chang CH, Kuo CK, Chen JH, Sorond F: Relation of C-reactive protein to stroke, cognitive disorders, and depression in the general population: systematic review and meta-analysis. Lancet Neurol 2005;4:371–380.

29 American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, ed 4. Washington, American Psychiatric Association, 1994.

30 Cummings JL, Mega M, Gray K, Rosenberg-Thompson S, Carusi DA, Gornbein J: The Neuropsychiatric Inventory: comprehensive assessment of psychopathology in dementia. Neurology 1994;44:2308–2314.

31 Montgomery SA, Åsberg M: A new depression scale designed to be sensitive to change. Br J Psychiatry 1979;134:382–389.

32 Blennow K, Wallin A, Ågren H, Spenger C, Siegfrid J, Vanmechelen E: Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer's Disease? Mol Chem Neuropathol 1995;26:231–245.

33 Vanmechelen E, Vanderstichele H, Davidsson P, Van Kerschaver E, Van der Perre B, Sjogren M, Andreasen N, Blennow K: Quantification of tau phosphorylated at threonine 181 in human cerebrospinal fluid: a sandwich ELISA with a synthetic phosphopeptide for standardization. Neurosci Lett 2000;285:49–52.

34 Andreasen N, Hesse C, Davidsson P, Minthon L, Wallin A, Winblad B, Vanderstichele H, Vanmechelen E, Blennow K: Cerebrospinal fluid β-amyloid (1–42) in Alzheimer disease and stability during the course of the disease. Arch Neurol 1999;56:673–680.

35 Boberg M, Croon LB, Gustafsson IB, Vessby B: Platelet fatty acid composition in relation to fatty acid composition in plasma and to serum lipoprotein lipids in healthy subjects with special reference to the linoleic acid pathway. Clin Sci 1985;68:581–587.

36 Hixson JE, Vernier DT: Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with H*hal*. J Lipid Res 1990;31:545–548.

37 Laws SM, Hone E, Gandy S, Martins RN: Expanding the association between the *APOE* gene and the risk of Alzheimer's disease: possible roles for *APOE* promoter polymorphisms and alterations in *APOE* transcription. J Neurochem 2003;84:1215–1236.

38 Cole GM, Morihara T, Lim GP, Yang F, Begum A, Frautschy SA: NSAID and antioxidant prevention of Alzheimer's disease: lessons from in vitro and animal models. Review. Ann NY Acad Sci 2004;1035:68–84.

39 Feldman H, Jacova C: Primary prevention and delay of onset of AD and dementia. Review. Can J Neurol Sci 2007;34:S84–S89.

40 Venkatraman JT, Chu WC: Effects of dietary omega-3 and omega-6 lipids and vitamin E on serum cytokines, lipid mediators and anti-DNA antibodies in a mouse model for rheumatoid arthritis. J Am Coll Nutr 1999; 18:602–613.

41 Garlind A, Brauner A, Höjeberg B, Basun H, Schultzberg M: Soluble interleukin-1 receptor type II levels are elevated in cerebrospinal fluid in Alzheimer's disease patients. Brain Res 1999;826:112–116.

42 Jia JP, Meng R, Sun YX, Sun WJ, Ji XM, Jia LF: Cerebrospinal fluid tau, Aβ1–42 and inflammatory cytokines in patients with Alzheimer's disease and vascular dementia. Neurosci Lett 2005;383:12–16.

43 Zuliani G, Ranzini M, Guerra G, Rossi L, Munari MR, Zurlo A, Volpato S, Atti AR, Ble' A, Fellin R: Plasma cytokines profile in older subjects with late onset Alzheimer's disease or vascular dementia. J Psychiatr Res 2007; 41:686–693.

44 Ray S, Britschgi M, Herbert C, Takeda-Uchimura Y, Boxer A, Blennow K, Friedman LF, Galasko DR, Jutel M, Karydas A, Kaye JA, Leszek J, Miller BL, Minthon L, Quinn JF, Rabinovici GD, Robinson WH, Sabbagh MN, So YT, Sparks L, Tabaton M, Tinklenberg J, Yesavage JA, Tibshirani R, Wyss-Coray T: Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. Nat Med 2007;13: 1359–1362.

45 Tabet N: Acetylcholinesterase inhibitors for Alzheimer's disease: anti-inflammatories in acetylcholine clothing! Age Ageing 2006;35: 336–338.

46 Reale M, Iarlori C, Gambi F, Lucci I, Salvatore M, Gambi D: Acetylcholinesterase inhibitors effects on oncostatin-M, interleukin-1β and interleukin-6 release from lymphocytes of Alzheimer's disease patients. Exp Gerontol 2005;40:165–171.

47 Kotani S, Sakaguchi E, Warashina S, Matsukawa N, Ishikura Y, Kiso Y, Sakakibara M, Yoshimoto T, Guo J, Yamashima T: Dietary supplementation of arachidonic and docosahexaenoic acids improves cognitive dysfunction. Neurosci Res 2006;56:159–164.

48 Yehuda S, Rabinovitz S, Carasso RL, Mostofsky DI: Essential fatty acids preparation (SR-3) improves Alzheimer's patients quality of life. Int J Neurosci 1996;87:141–149.

49 NIH website. ClinicalTrials.gov. DHA an omega 3 fatty acid in slowing the progression of Alzheimer's disease 2008.

50 Andreasen N, Minthon L, Clarberg A, Davidsson P, Gottfries J, Vanmechelen E, Vanderstichele H, Winblad B, Blennow K: Sensitivity, specificity and stability of CSF-tau in AD in community-based patient sample. Neurology 1999;53:1488–1494.

51 Andersson C, Blennow K, Almkvist O, Andreasen N, Engfeldt P, Johansson SE, Lindau M, Eriksdotter Jönhagen M: Increasing CSF phospho-tau levels during cognitive decline and progression to dementia. Neurobiol Aging 2008;29:1466–1473.

52 Oprica M, Hjorth E, Spulber S, Popescu BO, Ankarcrona M, Winblad B, Schultzberg M: Studies on brain volume, Alzheimer-related proteins and cytokines in mice with chronic overexpression of IL-1 receptor antagonist. J Cell Mol Med 2007;11:810–825.

53 Lindberg C, Chromek M, Ahrengart L, Brauner A, Schultzberg M, Garlind A: Soluble interleukin-1 receptor type II, IL-18 and caspase-1 in mild cognitive impairment and severe Alzheimer's disease. Neurochem 2005;46:551–557.

54 Kuhn PH, Marjaux E, Imhof A, De Strooper B, Haass C, Lichtenthaler SF: Regulated intramembrane proteolysis of the interleukin-1 receptor II by α-, β-and χ-secretase. J Biol Chem 2007;282:11982–11995.

IV

Effects of omega-3 fatty acids on microglial phagocytosis and inflammatory phenotype

[a] **Hjorth E,** [d] **Vedin I,** [d] **Palmblad J,** [e] **Cederholm T,** [b] **Freund-Levi Y,** [c] **Faxen-Irving G,** [b] **Wahlund L-O,** [f] **Basun H,** [b] **Eriksdotter-Jönhagen M and** [a] **Schultzberg M**

Department of Neurobiology, Care Sciences and Society, Divisions of [a]Neurodegeneration, [b]Clinical Geriatrics and [c]Nutrition, and [d]Department of Medicine, Karolinska Institutet, SE-141 86 Stockholm, Sweden. Department of Public Health and Caring Sciences, Divisions of [e]Clinical Nutrition and Metabolism, and [f]Geriatrics, Uppsala University Hospital, Uppsala Sweden

Abstract

Omega-3 fatty acids have been suggested to have beneficial effects on patients suffering from Alzheimer's disease (AD). With the aim to better understand the effects of the omega-3 fatty acid docosahexanoic acid (DHA) on neuroinflammation related to AD, human CHME3 microglia were incubated with DHA, alone or together with $A\beta_{1-42}$ or latex beads. The influence of inflammation induced by IL-1β was also investigated. It was found that DHA could stimulate phagocytosis of $A\beta_{1-42}$ but not latex beads. Furthemore, DHA was shown to decrease the secretion of several T_{H1} and T_{H2} cytokines. DHA at high concentrations also reduced the levels of brain-derived neurotrophic factor (BDNF). High concentrations of DHA also reduced microglial viability. Analysis of phagocytic cells suggested that phagocytosis of latex beads and $A\beta_{1-42}$ are performed by different mechanisms or by microglia of different phenotypes.

Key words: Alzheimer, amyloid, BDNF, brain-derived neurotrophic factor, cytokine, docosahexanoic acid, interleukin, omega-3

Introduction

Omega-3 fatty acids (O3:s) constitute a popular food supplement that has been ascribed several health benefits. Among the health conditions that O3:s are described as having a positive effect on are rheumatoid arthritis [1,2], cancer [3,4] and cardiovascular diseases [5,6]. In addition, there is increasing evidence indicating a positive effect of O3:s on disorders of the central nervous system (CNS) [7-10]. One of the most common neurodegenerative disorders is Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder that is characterized by the impairment and death of neurons, increased levels of the amyloid-β (Aβ) peptide [11], increased presence of intracellular tangles composed of a hyperphosphorylated form of the microtubule protein tau [12], and inflammation [13-15]. Although there is still controversy regarding the importance of Aβ and hyperphosphorylated tau for the inititation and progression of AD [16] it is believed that increased levels of Aβ are one of the culprits for the disease. Aβ is produced by intramembraneus cleavage of the amyloid precursor protein (APP), yielding a peptide which usually is 40 or 42 amino acids long, although both longer and shorter variants exist [17,18]. $A\beta_{1-42}$, and to a lesser degree $A\beta_{1-40}$, are prone to aggregation thus forming oligomers, fibrils and plaques [19]. The monomers and the different aggregational forms have been implicated in neurotoxicity and impairment in a plethora of studies [20]. Recently, the toxicity of dimers and trimers of Aβ has been considered [21,22], and it may be hypothesized that these forms are responsible for the neurotoxic effects and that the plaques may be relatively inert, or even beneficial, as they bind Aβ molecules, thereby preventing them from attaining neurotoxic configurations. On a clinical level, AD is characterized by cognitive deficits that initially affect learning and memory, but later in the disease are manifested by a global cognitive decline. The clinical symptoms mirror the pathological changes in the brain where the neuronal loss and plaque/tangle pathology begin in the memory-related areas (entorhinal cortex, hippocampus) and then spread to other parts of the cortex [23,24].

Docosahexanoic acid (DHA) is the O3 that has received most attention for its beneficial effects on factors related to AD [25-27]. To our knowledge, there is only one completed intervention study in which AD-patients have been treated with O3-supplements [28]. There was no effect on cognition, except in a subgroup of patients with very mild symptoms of AD, suggesting the importance of early intervention. The disorders in which O3:s have been reported as being beneficial have in common a component of inflammation. Inflammation is

prominent in AD as evidenced by activated microglia and astrocytes [29] and increased levels of pro-inflammatory cytokines in the AD brain [14,15]. It is unclear if inflammation is an original driving force in the pathogenesis of AD or a consequence of the disease. Inflammation has likely evolved for the body to effectively remove pathogens in the acute stage and after this is accomplished, promote tissue healing and repair in the resolving phase. However, in the case of chronic inflammatory states, such as in AD, the detrimental and tissue-damaging activities of inflammation are dominating [30]. Other evidence for the detrimental involvement of inflammation in AD is that patients under treatment with non-steroidal anti-inflammatory drugs (NSAID:s) have been shown to be protected against developing AD [31]. On a mechanistic level, Aβ has been shown to induce inflammation [32-35] and inflammation has been shown to increase the production of Aβ [36,37]. It has not been proven whether this reciprocal relationship is relevant for the progression of the disease, but a scenario with a self-reinforcing vicious circle is apparent.

There are also positive effects of inflammation that can help in healing the tissue. Secretion of neurotrophic growth factors from activated microglia is one activity related to inflammation that can protect and improve the function of neurons [38-41]. Another important activity of microglia is the ability to remove, phagocytose, pathogens and debris from the tissue. Phagocytosis of Aβ by monocytes and microglia has been shown in several studies [42-45]. To induce phagocytosis of Aβ, *i.e.* removal of a presumed pathogen without evoking the damaging activities of inflammation would be a promising therapy for AD. Ideally, beneficial activities such as microglial secretion of growth factors should also be increased, or at least not be decreased by such a therapy. DHA has been shown to downregulate inflammatory proteins *in vitro* [46,47] and *in vivo* [48]. DHA is also the precursor for the neurotrophic fatty acid neuroprotectin D1 [25] and is also an important cell membrane component. It is not known which of these activities are responsible for the proposed neuroprotective properties of DHA. Considering the widespread use of DHA-containing supplements and the urgent need for therapies to AD, it is of importance to investigate the effects of DHA not only on the traditional inflammatory markers but also on phagocytic activity and growth factor secretion to help characterize the effects of DHA on AD pathology. This may lead to better treatment and prevention strategies for AD, a disease which takes a heavy toll on society in terms of human suffering and care costs. To help realize this goal, we performed a series of experiments on a human microglia cell line (CHME3), in which the effects of DHA were

analysed with regard to phagocytosis of $A\beta_{1-42}$, expression of cellular markers, secretory products and cell viability.

Materials and methods

Chemicals

Docosohexanoic acid was purchased from Nucheckprep (Elysian, USA). $A\beta_{1-42}$ conjugated with HiLyteFluor488 or biotin was obtained from Anaspec (Fremont, USA). Latex beads conjugated with Texas Red were bought from Sigma, Stockholm, Sweden. Dimethylsulfoxide (DMSO), Triton-X100, bovine serum albumin (BSA), and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were also purchased from Sigma, Stockholm, Sweden. Normal donkey and goat serum, and fluorescence mounting medium (DakoPatts, Stockholm, Sweden). Streptavidin-7-amino-4-methyl-3-coumarinylacetic acid (AMCA) (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Lactate deydrogenase (LDH)-assay (Roche, Stockholm, Sweden). ELISA-kits for interleukin (IL) -6 and brain-derived neurotrophic factor (BDNF) (R&D systems, Abingdon, United Kingdom). 10-plex Th1/Th2 cytokine assay (Meso Scale Diagnostics, Gaithersburg, USA). Cell culture medium, phosphate-buffered saline (PBS), GlutaMaxII, foetal calf serum (FCS) and PBS-based enzyme-free cell dissociation buffer (Invitrogen, Stockholm, Sweden). Cell culture bottles and multi-well plates (BD Biosciences, Stockholm, Sweden).

Cell cultures

Human microglial cells (CHME3) were obtained as a kind gift from Prof. M Tardieu, Neurologie pédiatrique, Hôpital Bicêtre, Assistance publique, Hôpitaux de Paris, Paris, France. CHME3 cells were cultured in T75 or T175 bottles in culture medium (DMEM/high glucose w/o sodium pyruvate supplemented with GlutaMaxII and 10% heat-inactivated FCS). The cells were subcultured at confluence using enzyme-free cell dissociation buffer after washing once with phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+}.

Experimental procedures

The CHME3 microglial cells were seeded in 48-well plates for analysis of cell viability (MTT) and cytotoxicity (LDH) (see Supplementary material) and in 6-well plates for flow-cytometry and analysis of secretory products. For the experiments on latex beads 10 cm petri dishes were used. All experiments were performed at a confluence of ~60-70%. $A\beta_{1-42}$ was dissolved in DMSO to a concentration of 5 mg/ml and stored in darkness at +4^{o}C until use at a final concentration of 1 μg/ml for all experiments. DHA was diluted in 95% EtOH to a concentration of 200 mM and stored in a nitrogen atmosphere. For experiments, the cells were washed with serum-free medium and then treated with $A\beta_{1-42}$ or vehicle (DMSO) together with DHA or vehicle (95% EtOH), also in serum-free medium. For analyzing phagocytosis of latex beads the cells were washed with serum-free medium and latex beads were added in a 1:4000 dilution together with vehicle, 0.05 μM DHA, 50 ng/ml IL-1β or 0.05 μM DHA together with 50 ng/ml IL-1β in serum-free medium. After 6, 24, and 48 h of incubation, the cultures were analyzed for uptake of $A\beta_{1-42}$, expression of cellular markers and secretory products. Analysis of cultures incubated with latex beads were performed at 48 h. Cell viability and cell death were analysed at 24 h.

Quantification of $A\beta_{1-42}$/latex bead phagocytosis and cellular markers by flow-cytometry

At 6, 24 and 48 h after starting the experiment, the CHME3 microglial cells were dissociated with PBS-based enzyme-free dissociation buffer, and centrifuged at 1500 x g for 10 min. The cells were then resuspended and fixed in 300 μl of 1% para-formaldehyde (PF) in PBS, for 40 min at room temperature in a 12 ml tube. The cells were then washed by addition of 10 ml PBS followed by centrifugation at 1500 x g for 10 min, removal of supernatant, resuspension in 300 ml of PBS and then stored at +4° C in darkness.

For analysis of cellular uptake of $A\beta_{1-42}$ and the expression of cellular markers, a volume of cell suspension was added to the same volume of a 2X PBS solution with a primary antibody to human IL-1β made in rabbit (1:200; gift from Dr. Stefan Svensson, Statens Bakteriologiska Laboratorium, Stockholm, Sweden), 10% normal donkey serum and 0.2% Triton X-100 (Sigma, Sweden) in a 1.5 ml tube. The cells were incubated as such overnight after which they were washed with 1 ml PBS followed by centrifugation at 2500 x g for 20 min. A volume of the resulting cell suspension was then incubated for 1 h RT with the same volume

of a 2X PBS solution with a NL637-conjugated donkey-anti-rabbit secondary antibody (1:500; R&D systems, London, England), 1 μg/ml propidium iodide (PI) and 20 % bovine serum albumin (Sigma, Sweden). Negative control for unstained cells (unstained control) was a cell suspension from the vehicle group incubated with solutions without primary and secondary antibodies. Negative control for the primary antibody (primary antibody control) was a cell suspension from the vehicle groups incubated with a solution without primary antibody followed by incubation with the secondary antibody. The cells were analysed immediately after incubation while the tubes not in use were kept at +4° C and in darkness.

PI was used in conjunction with gating through the front-scatter (FSC) and side-scatter (SSC) plot to differentiate cells from debris. Fixation with PF renders cells permeable to PI and therefore the nuclei of all fixed cells will be stained with PI while debris will remain unstained. The background signal in the PI-wavelength was established by analyzing the unstained control and setting the limit of detection according to this signal. Events thus detected and gated by the flow-cytometer was further analysed in a quadrant plot for the presence of $A\beta_{1-42}$ and IL-1β. A cell positive for phagocytosis of $A\beta_{1-42}$ ($A\beta_{1-42}$+) was defined as a cell that produced a signal in the HyliteFluor488-wavelength that was higher than the signal from the cells not incubated with $A\beta_{1-42}$. Positive signal for IL-1β (IL-1β+) was defined as a cell producing a signal higher than the signal produced by the primary antibody control. By setting up the quadrant border in this way it is possible to determine if a cell is negative for both $A\beta_{1-42}$ and IL-1β ($A\beta_{1-42}$-/IL-1β-), negative for $A\beta_{1-42}$ and positive for IL-1β ($A\beta_{1-42}$-/IL-1β+), positive for $A\beta_{1-42}$ and negative for IL-1β ($A\beta_{1-42}$+/IL-1β-) or positive for both $A\beta_{1-42}$ and IL-1β ($A\beta_{1-42}$+/IL-1β+). Analysis of phagocytosis of latex beads were performed in a similar way, except for the use of acridine orange (AO) as a nuclear stain.

Enzyme-linked immunosorbent assay (ELISA) and multiplex Th1/Th2 cytokine assay

The levels of IL-6 and BDNF in the cell culture medium were analyzed with commercially available ELISA-kits according to the manufacturer's instructions. Analysis of optical density (OD) was performed in a TECAN Safire2 plate reader. The levels of interferon-γ (IFN-γ), IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-8 and tumour necrosis factor-α (TNF-α), were analysed with a multiplex Th1/Th2 human cytokine kit according to the manufacturer's instructions in a Meso Scale Diagnostics Model 1200 plate reader.

Results

Cell viability and cell death

To investigate the cytotoxic potential of DHA on CHME3 human microglia, the cells were incubated with 1 and 10 μM DHA for 48 h. Microscopical inspection of the cultures indicated severe cellular stress (rounded-up and detached cells) in the cultures treated with 10 μM DHA. Analysis with MTT and LDH assays indicated decreased cell viability and increased cell death in the cultures treated with this dose (Fig. 1). The results were not statistically significant which may be due to the low number of experiments (n=4) in these analyses. However, the microscopical observations and the fact that the results were at least suggestive of cell death or stress cautioned for using concentrations of 10 μM.

Effects of DHA on uptake of $A\beta_{1-42}$

Analysis by flow-cytometry of the uptake of $A\beta_{1-42}$ by the CHME3 microglia revealed a significant stimulatory effect of DHA at 6 h. The concentration of 0.05 μM DHA resulted in a 23% ($p < 0.05$) increase in the uptake as compared with control ($A\beta_{1-42}$ alone), and with 0.5 μM there was an increase of 18% ($p < 0.05$) (Fig. 2). The stimulatory effect appeared to linger at 24 h although the results were void of significance, while at 48 h the degree of uptake was similar in all treatments.

Effects of DHA on uptake of latex beads

There was no significant effect on the uptake of latex beads by any treatment as analyzed with flow-cytometry.

Immunoreactivity to IL-1β and iNOS and the relation to phagocytosis of $A\beta_{1-42}$

The phenotype of microglial cells with and without uptake of $A\beta_{1-42}$ was analysed by flow-cytometry with regard to immunoreactivity to IL-1β and iNOS (Fig. 3A-B) in the different

treatment groups. Incubation with 0.5 or 2.5 μM DHA for 24 h resulted in an increase in the number of IL-1β+ microglia (Fig. 3A). At 0.5 μM the increase was 60% ($p < 0.01$) as compared with vehicle, while 2.5 μM increased IL-1β immunoreactivity by 21% ($p < 0.05$). At 48 h, the immunoreactivity to IL-1β was similar in all groups.

The immunoreactivity to iNOS appeared to increase after 48 h of incubation with DHA although the only significant increase was observed with 0.05 μM DHA (Fig. 4). There were no significant changes in immunoreactivity to IL-1β or iNOS seen upon incubation of the microglia with $A\beta_{1-42}$ alone, or with $A\beta_{1-42}$ together with DHA.

To investigate the phenotype of the microglia displaying phagocytosis of $A\beta_{1-42}$, the phagocytic and non-phagocytic populations of microglia in each treatment were compared with regard to the proportion of cells with immunoreactivity to IL-1β (Fig. 4A) and iNOS (Fig. 4B). Analysis of treatment with $A\beta_{1-42}$ alone showed that a significantly larger proportion of phagocytic cells were immunoreactive to IL-1β at 6 h ($p < 0.00005$) and at 48 h ($p < 0.05$). Microglia treated with DHA together with $A\beta_{1-42}$ showed the same pattern, *i.e.* significantly more phagocytic cells were immunoreactive to IL-1β at 6, 24 and 48 h in all concentrations tested.

Analysis of the differential immunoreactivity to iNOS at 24 and 48 h showed that there were significantly more cells displaying immunoreactivity to iNOS in the phagocytic population at 48 h. However, when DHA was added together with $A\beta_{1-42}$, the difference was abolished (Fig. 4B). At 24 h the treatment with 0.05 DHA together with $A\beta_{1-42}$ induced a phagocytic population with significantly higher expression of iNOS.

Secretion of BDNF and IL-6 from microglial cultures incubated with $A\beta_{1-42}$

The secreted levels of BDNF and IL-6 were analysed with ELISA (Fig. 5). The incubation with $A\beta_{1-42}$ alone did not affect the levels of BDNF and IL-6 as compared with vehicle. There were no significant differences in the secreted levels of IL-6 upon treatment with DHA. Incubation with 0.01 μM DHA together with $A\beta_{1-42}$ decreased the levels of BDNF in the medium with 53% of vehicle at 24 h ($p < 0.001$), and incubation with 2.5 μM DHA at 48 h decreased the levels of BDNF in the medium to 74% of vehicle ($p < 0.001$).

Secretion of Th1 and Th2 cytokines from microglial cultures incubated with latex beads

The secretion of a set of Th1/Th2 cytokines (IFNγ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-8 and TNFα) at 48 h of incubation was analyzed with Meso Scale Diagnostics multiplex technology (Fig. 6). All cytokines were present at detectable levels in the conditioned medium. The incubation with 0.05 μM DHA decreased the levels of IL-12p70 to 51% of vehicle ($p < 0.05$). The same treatment also decreased the levels of IL-13 (58% of vehicle), IL-1β (50% of vehicle) and IL-8 (75% of vehicle) ($p < 0.05$ in all cases). Treatment with 0.05 μM DHA together with 50 ng/ml IL-1β reduced the levels of TNFα to 48% of vehicle ($p < 0.05$), and the levels of IL-1β itself to 5% of vehicle ($p < 0.05$).

Secretion of BDNF and IL-6 from microglial cultures incubated with latex beads

The levels of secreted BDNF and IL-6 were measured with ELISA (Fig. 7). We found that 50 ng/ml IL-1β increased the secretion of BDNF by 38% compared to vehicle ($p < 0.01$). IL-1β increased the secreted levels of IL-6 with more than 20-fold ($p < 0.05$). Co-incubation with 0.05 μM DHA and 50 ng/ml IL-1β increased the secreted levels of IL-6 15-fold as compared to vehicle ($p < 0.05$), and to DHA alone ($p < 0.05$).

Immunoreactivity to IL-1β, IL-1RI, TNFα, IL-4R, iNOS and IL-6 and the relation to phagocytosis of latex beads

There were no significant differences in immunoreactivity between the treatment groups when analyzing the microglia by flow-cytometry with regard to the expression of IL-1β, IL-1RI, TNFα, IL-4R, iNOS and IL-6 at 48 h incubation with latex beads. When analyzing the differential expression of cellular markers we found that the cells showing phagocytosis of latex beads had a significantly lower expression of iNOS in all treatment groups ($p < 0.05$ in all cases) (Fig. 8). Treatment with 0.05 μM DHA slightly decreased the number of phagocytic cells displaying immunoreactivity to TNFα. Treatment with 0.05 μM DHA together with 50 ng/ml IL-1β increased the number of phagocytic cells displaying immunoreactivity to IL-6 with ($p < 0.05$).

Discussion

In the present study, we provide data that both support and contradict the hypothesis that DHA can protect neurons in a setting of AD-like pathology. DHA was found to have stimulatory effects on phagocytosis of $A\beta_{1-42}$ and to exert inhibitory effects on the secretion of pro-inflammatory cytokines and chemokines that have been suggested to be a part of, or worsen, the pathological changes in the AD-afflicted brain. On the other hand, high concentrations of DHA were found to decrease the secretion of the neuroprotective growth factor BDNF. DHA also decreased the levels of anti-inflammatory cytokines. Anti-inflammatory cytokines, such as IL-4, have been suggested to be neuroprotective [49,50] and stimulatory on phagocytosis [51-53]. There was no effect on phagocytosis of latex beads by DHA or IL-1β, or by their combination. In a previous study we could not detect any effect on phagocytosis of $A\beta_{1-42}$ by IL-1β [54].

Little is known about the effects of DHA on human microglia. To our knowledge, the present study is the first to examine the effects of DHA, or any O3, on phagocytosis by microglia, derived from any species. A few studies have been performed to investigate the effects by DHA on phagocytosis by monocytes, which have a similar repertoire of responses as microglia. Monocytes are believed to enter the CNS and then differentiate to resident microglia [55] and the comparison is therefore relevant. It has been suggested that phagocytosis of $A\beta_{1-42}$ is mainly performed by monocytes newly entered into the CNS and that phagocytosis by resident microglia is secondary to the action of the migrated monocytes [56].

A study by Halvorsen et al showed that diet supplementation of highly purified DHA did not affect phagocytosis of E. Coli by human monocytes collected and isolated from the participants in the study [57]. In contrast, a study by Gorjão et al showed an increase in the phagocytosis of Zymosan A particles by monocytes isolated from blood collected from human subjects treated with fish-oil capsules [58]. Since the capsules contained eicosapentaenoic acid (EPA) in addition to DHA, and also other constituents not mentioned in the study, a comparison with the present study is difficult, except for the conclusion that DHA by itself may not be optimal for stimulating phagocytosis. As the data from this study also suggest, the nature of the object being phagocytosed, may also modulate the profile of induced activation. It should also be mentioned that in the study by Gorjão et al, there was evidence of pro-inflammatory activation of the monocytes including a large increase in the

production of reactive oxygen species, IFNγ and TNFα, and decreased IL-2 and IL-4 production. This activation contradicts the results obtained by others, where O3-supplementation produced an anti-inflammatory effect [59,60].

We have observed a negative effect of $A\beta_{1-42}$ on the microglial secretion of BDNF in previous studies [54]. In the present study, the reduction in the secretion of BDNF in microglial cultures treated with $A\beta_{1-42}$ was just void of significance at 24 h. However, when $A\beta_{1-42}$ was added together with 0.01 mM DHA, there was a significant reduction in BDNF at 24 h. In cultures treated without $A\beta_{1-42}$ the impression was that DHA stimulated the secretion of BDNF, although the results were not statistically significant. At 48 h after adding the substances, the highest concentration of DHA 2.5 (μM) reduced the secretion of BDNF significantly to 60% of the levels observed upon treatment with vehicle.

One mechanism that has been suggested to mediate the effects of DHA is the production of anti-inflammatory and neuroprotective fatty acids: neuroprotectins and maresins, for which DHA is the precursor [25,61]. Maresins are suggested to be involved in the resolving phase of inflammation, when phagocytosis is abundant and an anti-inflammatory state is dominating in the tissue (for example, [62]). Interestingly, maresin R1 has been shown to increase phagocytosis of Zymosan A particles by human monocytes [61]. The stimulatory effect on phagocytosis of $A\beta_{1-42}$ by DHA was observed after 6 h of incubation and appeared to dissipate with time. Fatty acids easily become oxidized and DHA and its downstream effectors could disappear from the culture in this way at an early time point, having a limited effect with time. It can also be hypothesized that other cell types in the brain, apart from microgli, are the main responsible for converting DHA into active substances such as maresins, due to a higher expression of the biosynthetic enzymes. Other factors such as the impact of cell membrane composition and fluidity have also been suggested as mediators of the effects of DHA regarding AD-related impairment in cognition [63], and may also play a role in modulating the responses of immune cells.

In this study, we observed signs of inflammatory activation of human microglia after treatment with DHA in the form of an increase in the number of IL-1β+ cells and a slight increase in IL-6 secretion. However, when the CHME3 microglia were incubated with IL-1β there was a lower number of cells that displayed uptake of $A\beta_{1-42}$ compared to the vehicle group at 48 h. At this time point the levels of IL-6 in the cultures were 20-fold higher upon treatment with IL-1β, indicating an inflammatory activation by IL-1β. This may indicate that

cells secreting IL-1β decrease their cellular levels of this cytokine, which makes sense under the assumption that the process of secretion is working at a faster pace than the translatory machinery. Adding to the credibility of this explanation is the relatively low cellular level of IL-6 in cultures treated with IL-1β. The overall impression is that DHA decreased microglial activation as shown by a reduction in the secreted levels of both pro- and anti-inflammatory cytokines. Another indication of an anti-inflammatory effect of DHA is that the increase in the number of iNOS+/$A\beta_{1-42}$+ cells in the cultures treated with $A\beta_{1-42}$ alone, was abolished when DHA was added together with $A\beta_{1-42}$. Interestingly, this effect was not seen when DHA was added to cells incubated with latex beads where the pattern was reversed; cells displaying phagocytosis of latex beads were significantly less prone to express iNOS. This discrepancy hints at different pathways of uptake for latex beads and $A\beta_{1-42}$. We conclude that DHA would not cause deleterious effects and that it may even reduce the pathology by alleviating the inflammation and increasing the phagocytosis of the pathogenic $A\beta_{1-42}$ peptide. It should be kept in mind, however, that the downstream effects of DHA are dependent on the expression of enzymes and of the receptors for the DHA-derived products, which is a new field in medicine. Further studies on these aspects are necessary to produce data that can be translated into therapies for AD.

Acknowledgements

The support of this work by grants from the Swedish Research Council (072194), Swedish Brain Power, The Knut and Alice Wallenberg Foundation, Karolinska Institutet Research funds, Stiftelsen för Gamla Tjänarinnor, Alzheimer Foundation Sweden, Gun och Bertil Stohnes stiftelse, and Stockholm County Council, are gratefully acknowledged.

Figure legends

Fig. 1 Effects of docosahexanoic acid (DHA) on cell viability and cell death of human CHME3 microglial cells. The cells were incubated with DHA at 1 and 10 μM for 48 h after which they were analysed with MTT and LDH assays. The data from the MTT-assay are expressed as % of control (vehicle) set at 100%, while the data from the LDH-assay are expressed as % of positive control (vehicle-treated cells lysed with Triton-X100) and shown as median ± percentiles (25% - 75% and 10% - 90%).

Fig. 2. Effects of docosahexanoic acid (DHA) on the uptake of $A\beta_{1-42}$ in human CHME3 microglial cells. The cells were incubated with 1 μg/ml $A\beta_{1-42}$, alone or together with 0.01 – 2.5 μM DHA for 6, 24 and 48 h, after which they were harvested and fixed. The cells were then analysed by flow-cytometry for the uptake of $A\beta_{1-42}$. The data are expressed as % uptake of $A\beta_{1-42}$ set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by * ($p < 0.05$).

Fig 3A-B. Effects of docosahexanoic acid (DHA), alone or together with $A\beta_{1-42}$, on immunoreactivity to interleukin (IL)-1β and inducible nitric oxide synthase (iNOS) in human CHME3 microglial cells. The cells were incubated with 1 μg/ml $A\beta_{1-42}$, alone or together with 0.01 – 2.5 μM DHA for 6, 24 and 48 h, after which they were harvested, fixed and subjected to indirect immunocytochemistry for IL-1β (A) and iNOS (B). The data are expressed as % immunoreactivity upon incubation with $A\beta_{1-42}$ alone set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by * ($p < 0.05$) and ** ($p < 0.01$).

Fig. 4A-B. Differential expression of interleukin (IL)-1β and inducible nitric oxide synthase (iNOS) by human CHME3 microglia in the populations of cells displaying ($A\beta_{1-42}$+) or not displaying ($A\beta_{1-42}$-) phagocytosis of $A\beta_{1-42}$ after treatment with docosahexanoic acid (DHA) (0.01 – 2.5 μM) together with 1 μg/ml $A\beta_{1-42}$. After 6, 24 and 48 h of exposure to $A\beta_{1-42}$, the cells were fixed, subjected to immunocytochemistry, and analysed by flow-cytometry. The

number of IL-1β+ (A) or iNOS+ (B) cells in the $A\beta_{1-42}$+ population was compared with the corresponding proportion in the $A\beta_{1-42}$- population of cells. The comparison was performed in each treatment group, using the Wilcoxon Matched Pairs Test. The data are shown as median ± percentiles (25% - 75% and 10% - 90%), n = 7. Statistical difference in immunoreactivity between $A\beta_{1-42}$-negative/positive cells is indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Fig 5. Effects of docosahexanoic acid (DHA), alone or together with $A\beta_{1-42,}$ on the secretion of brain-derived neurotrophic factor (BDNF) from human CHME3 microglia. The cells were incubated with 1 μg/ml $A\beta_{1-42}$, alone or together with 0.01 – 2.5 μM DHA for 24 and 48 h. The data are expressed as % of control vehicle set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by *** ($p < 0.001$).

Fig 6. Effects of docosahexanoic acid (DHA), alone or together with interleukin (IL)-1β, on the secretion of IL-12p70, IL-13, IL-1β, IL-8 and tumour necrosis factor-α (TNF-α) from human CHME3 microglia. The cells were incubated with 0.05 μM DHA and 50 ng/ml IL-1β, alone or in combination, for 48 h after which the medium was collected and analysed with Meso Scale Diagnostics multiplex technology for the levels of a set of Th1/Th2 cytokines. The data are expressed as % of control vehicle set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by * ($p < 0.05$).

Fig 7. Effects of docosahexanoic acid (DHA) and interleukin (IL)-1β incubated with latex beads on the secretion of IL-6 and brain-derived neurotrophic factor (BDNF) from human CHME3 microglia. The cells were incubated with latex beads together with vehicle, docosahexanoic acid (DHA) (0.05 μM), IL-1β (50 ng/ml), or DHA (0.05 μM) together with IL-1β (50 ng/ml). After 48 h of treatment, the culture medium was collected and analysed with ELISA for the levels of IL-6 and BDNF. The data are expressed as % of control vehicle

set at 100%, and shown as median ± percentiles (25% - 75% and 10% - 90%). Statistical difference from control is indicated by * ($p < 0.05$).

Fig 8A-B. Differential expression of interleukin (IL)-6 and inducible nitric oxide synthase (iNOS) by human CHME3 microglia in the populations of cells displaying or not displaying phagocytosis of latex beads after treatment with vehicle, docosahexanoic acid (DHA) (0.05 µM), IL-1β (50 ng/ml), or DHA (0.05 µM) together with IL-1β (50 ng/ml). After 48 h of exposure the cells were fixed, subjected to immunocytochemistry, and analysed by flow-cytometry. The proportion of cells positive to IL-6 (A) or iNOS (B) in the population of cells displaying phagocytosis of latex beads was compared with the corresponding proportion in the non-phagocytic population. The comparison was performed in each treatment group, using the Wilcoxon Matched Pairs Test. The data are shown as median ± percentiles (25% - 75% and 10% - 90%), n = 7. Statistical difference in immunoreactivity between $A\beta_{1-42}$-negative/positive cells is indicated by * ($p < 0.05$).

References

1. van der Tempel H, Tulleken JE, Limburg PC, Muskiet FA, van Rijswijk MH, Effects of fish oil supplementation in rheumatoid arthritis, *Annals of the Rheumatic Diseases*, 49:76-80, 1990
2. Kremer JM, Lawrence DA, Jubiz W, DiGiacomo R, Rynes R, Bartholomew LE, Sherman M, Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis. Clinical and immunologic effects, *Arthritis & Rheumatism*, 33:810-820, 1990
3. Karmali RA, Marsh J, Fuchs C, Effect of omega-3 fatty acids on growth of a rat mammary tumor, *Journal of the National Cancer Institute*, 73:457-461, 1984
4. Lowell JA, Parnes HL, Blackburn GL, Dietary immunomodulation: beneficial effects on oncogenesis and tumor growth, *Critical Care Medicine*, 18:S145-148, 1990
5. Kinsella JE, Effects of polyunsaturated fatty acids on factors related to cardiovascular disease, *American Journal of Cardiology*, 60:23G-32G, 1987

6. Connor WE, Effects of omega-3 fatty acids in hypertriglyceridemic states, *Seminars in Thrombosis & Hemostasis*, 14:271-284, 1988
7. Morris MC, Evans DA, Bienias JL, Tangney CC, Bennett DA, Wilson RS, Aggarwal N, Schneider J, Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease, *Archives of Neurology*, 60:940-946, 2003
8. Bousquet M, Saint-Pierre M, Julien C, Salem N, Jr., Cicchetti F, Calon F, Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease, *FASEB Journal*, 22:1213-1225, 2008
9. Nordvik I, Myhr KM, Nyland H, Bjerve KS, Effect of dietary advice and n-3 supplementation in newly diagnosed MS patients, *Acta Neurologica Scandinavica*, 102:143-149, 2000
10. Arvindakshan M, Ghate M, Ranjekar PK, Evans DR, Mahadik SP, Supplementation with a combination of omega-3 fatty acids and antioxidants (vitamins E and C) improves the outcome of schizophrenia, *Schizophrenia Research*, 62:195-204, 2003
11. Selkoe DJ, The deposition of amyloid proteins in the aging mammalian brain: implications for Alzheimer's disease, *Annals of Medicine*, 21:73-76, 1989
12. Wolozin BL, Pruchnicki A, Dickson DW, Davies P, A neuronal antigen in the brains of Alzheimer patients, *Science*, 232:648-650, 1986
13. Eikelenboom P, Rozemuller JM, Kraal G, Stam FC, McBride PA, Bruce ME, Fraser H, Cerebral amyloid plaques in Alzheimer's disease but not in scrapie-affected mice are closely associated with a local inflammatory process, *Virchows Archiv. B. Cell Pathology*, 60:329-336, 1991
14. Bauer J, Strauss S, Schreiter-Gasser U, Ganter U, Schlegel P, Witt I, Yolk B, Berger M, Interleukin-6 and alpha-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices, *FEBS Letters*, 285:111-114, 1991
15. Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, White CL, 3rd, Araoz C, Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease, *The Proceedings of the National Academy of Sciences*, 86:7611-7615, 1989
16. Lee VM, Biomedicine. Tauists and β-aptists united--well almost!, *Science*, 293:1446-1447, 2001
17. Welander H, Franberg J, Graff C, Sundstrom E, Winblad B, Tjernberg LO, Abeta43 is more frequent than Abeta40 in amyloid plaque cores from Alzheimer disease brains, *Journal of Neurochemistry*, 110:697-706, 2009
18. Wiltfang J, Esselmann H, Bibl M, Smirnov A, Otto M, Paul S, Schmidt B, Klafki HW, Maler M, Dyrks T, Bienert M, Beyermann M, Ruther E, Kornhuber J, Highly conserved and disease-specific patterns of carboxyterminally truncated Abeta peptides 1-37/38/39 in addition to 1-40/42 in Alzheimer's disease and in patients with chronic neuroinflammation, *Journal of Neurochemistry*, 81:481-496, 2002
19. Finder VH, Glockshuber R, Amyloid-beta aggregation, *Neurodegenerative Diseases*, 4:13-27, 2007
20. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW, Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state, *Journal of Neuroscience*, 13:1676-1687, 1993
21. Hung LW, Ciccotosto GD, Giannakis E, Tew DJ, Perez K, Masters CL, Cappai R, Wade JD, Barnham KJ, Amyloid-beta peptide (Abeta) neurotoxicity is modulated by the rate of peptide aggregation: Abeta dimers and trimers correlate with neurotoxicity, *Journal of Neuroscience*, 28:11950-11958, 2008
22. Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ, Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory, *Nature Medicine*, 14:837-842, 2008

23. Brun A, Englund E, Regional pattern of degeneration in Alzheimer's disease: neuronal loss and histopathological grading. A. Brun & E. Englund. Histopathology 1981; 5; 549-564, *Histopathology*, 41:37-38,
24. Bobinski M, Wegiel J, Wisniewski HM, Tarnawski M, Reisberg B, Mlodzik B, de Leon MJ, Miller DC, Atrophy of hippocampal formation subdivisions correlates with stage and duration of Alzheimer disease, *Dementia*, 6:205-210, 1995
25. Lukiw WJ, Cui JG, Marcheselli VL, Bodker M, Botkjaer A, Gotlinger K, Serhan CN, Bazan NG, A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease, *Journal of Clinical Investigation*, 115:2774-2783, 2005
26. Florent S, Malaplate-Armand C, Youssef I, Kriem B, Koziel V, Escanye MC, Fifre A, Sponne I, Leininger-Muller B, Olivier JL, Pillot T, Oster T, Docosahexaenoic acid prevents neuronal apoptosis induced by soluble amyloid-beta oligomers, *Journal of Neurochemistry*, 96:385-395, 2006
27. Green KN, Martinez-Coria H, Khashwji H, Hall EB, Yurko-Mauro KA, Ellis L, LaFerla FM, Dietary docosahexaenoic acid and docosapentaenoic acid ameliorate amyloid-beta and tau pathology via a mechanism involving presenilin 1 levels, *Journal of Neuroscience*, 27:4385-4395, 2007
28. Freund-Levi Y, Eriksdotter-Jonhagen M, Cederholm T, Basun H, Faxen-Irving G, Garlind A, Vedin I, Vessby B, Wahlund LO, Palmblad J, Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial, *Archives of Neurology*, 63:1402-1408, 2006
29. Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D, Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease, *Journal of Neuroimmunology*, 24:173-182, 1989
30. Diegelmann RF, Evans MC, Wound healing: an overview of acute, fibrotic and delayed healing, *Frontiers in Bioscience*, 9:283-289, 2004
31. McGeer PL, Schulzer M, McGeer EG, Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies, *Neurology*, 47:425-432, 1996
32. Araujo DM, Cotman CW, β-amyloid stimulates glial cells in vitro to produce growth factors that accumulate in senile plaques in Alzheimer's disease, *Brain Research*, 569:141-145, 1992
33. Meda L, Cassatella MA, Szendrei GI, Otvos L, Jr., Baron P, Villalba M, Ferrari D, Rossi F, Activation of microglial cells by beta-amyloid protein and interferon-gamma, *Nature*, 374:647-650, 1995
34. Combs CK, Karlo JC, Kao SC, Landreth GE, beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis, *Journal of Neuroscience*, 21:1179-1188, 2001
35. Takata K, Kitamura Y, Umeki M, Tsuchiya D, Kakimura J, Taniguchi T, Gebicke-Haerter PJ, Shimohama S, Possible involvement of small oligomers of amyloid-beta peptides in 15-deoxy-delta 12,14 prostaglandin J2-sensitive microglial activation, *Journal of Pharmacological Sciences*, 91:330-333, 2003
36. Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B, Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of $A\beta_{1-40}$ and $A\beta_{1-42}$ by human astrocytes, *Neurobiological Disorders*, 7:682-689, 2000
37. Grilli M, Ribola M, Alberici A, Valerio A, Memo M, Spano P, Identification and characterization of a κB/Rel binding site in the regulatory region of the amyloid precursor protein gene, *Journal of Biological Chemistry*, 270:26774-26777, 1995
38. Ide CF, Scripter JL, Coltman BW, Dotson RS, Snyder DC, Jelaso A, Cellular and molecular correlates to plasticity during recovery from injury in the developing mammalian brain, *Progress In Brain Research*, 108:365-377, 1996

39. Hutchinson AJ, Chou CL, Israel DD, Xu W, Regan JW, Activation of EP2 prostanoid receptors in human glial cell lines stimulates the secretion of BDNF, *Neurochemistry International*, 54:439-446, 2009
40. Lai AY, Todd KG, Hypoxia-activated microglial mediators of neuronal survival are differentially regulated by tetracyclines, *GLIA*, 53:809-816, 2006
41. Heese K, Hock C, Otten U, Inflammatory signals induce neurotrophin expression in human microglial cells, *Journal of Neurochemistry*, 70:699-707, 1998
42. Kakimura J, Kitamura Y, Taniguchi T, Shimohama S, Gebicke-Haerter PJ, Bip/GRP78-induced production of cytokines and uptake of amyloid-beta(1-42) peptide in microglia, *Biochemical & Biophysical Research Communications*, 281:6-10, 2001
43. Kakimura J, Kitamura Y, Takata K, Umeki M, Suzuki S, Shibagaki K, Taniguchi T, Nomura Y, Gebicke-Haerter PJ, Smith MA, Perry G, Shimohama S, Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins, *FASEB Journal*, 16:601-603, 2002
44. Weltzien RB, Pachter JS, Visualization of beta-amyloid peptide (Abeta) phagocytosis by human mononuclear phagocytes: dependency on Abeta aggregate size, *Journal of Neuroscience Research*, 59:522-527, 2000
45. Malm TM, Magga J, Kuh GF, Vatanen T, Koistinaho M, Koistinaho J, Minocycline reduces engraftment and activation of bone marrow-derived cells but sustains their phagocytic activity in a mouse model of Alzheimer's disease, *GLIA*, 56:1767-1779, 2008
46. De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA, Jr., Libby P, The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells, *Arteriosclerosis & Thrombosis*, 14:1829-1836, 1994
47. Khalfoun B, Thibault F, Watier H, Bardos P, Lebranchu Y, Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human endothelial cell production of interleukin-6, *Advances in Experimental Medicine & Biology*, 400B:589-597, 1997
48. Himmelfarb J, Phinney S, Ikizler TA, Kane J, McMonagle E, Miller G, Gamma-tocopherol and docosahexaenoic acid decrease inflammation in dialysis patients, *Journal of Renal Nutrition*, 17:296-304, 2007
49. Deboy CA, Xin J, Byram SC, Serpe CJ, Sanders VM, Jones KJ, Immune-mediated neuroprotection of axotomized mouse facial motoneurons is dependent on the IL-4/STAT6 signaling pathway in CD4(+) T cells, *Experimental Neurology*, 201:212-224, 2006
50. Clarke RM, Lyons A, O'Connell F, Deighan BF, Barry CE, Anyakoha NG, Nicolaou A, Lynch MA, A pivotal role for interleukin-4 in atorvastatin-associated neuroprotection in rat brain, *Journal of Biological Chemistry*, 283:1808-1817, 2008
51. Wirth JJ, Kierszenbaum F, Zlotnik A, Effects of IL-4 on macrophage functions: increased uptake and killing of a protozoan parasite (Trypanosoma cruzi), *Immunology*, 66:296-301, 1989
52. Sampson LL, Heuser J, Brown EJ, Cytokine regulation of complement receptor-mediated ingestion by mouse peritoneal macrophages. M-CSF and IL-4 activate phagocytosis by a common mechanism requiring autostimulation by IFN-beta, *Journal of Immunology*, 146:1005-1013, 1991
53. Capsoni F, Minonzio F, Ongari AM, Carbonelli V, Galli A, Zanussi C, IL-10 up-regulates human monocyte phagocytosis in the presence of IL-4 and IFN-gamma, *Journal of Leukocyte Biology*, 58:351-358, 1995
54. **Hjorth E** FD, Weiner H, Schultzberg M, Effects of immunomodulatory substances on phagocytosis of Abeta1-42 by human microglia, *International Journal of Alzheimer's disease*, Accepted, 2010
55. Ling EA, Transformation of monocytes into amoeboid microglia in the corpus callosum of postnatal rats, as shown by labelling monocytes by carbon particles, *Journal of Anatomy*, 128:847-858, 1979

56. Frenkel D, Puckett L, Petrovic S, Xia W, Chen G, Vega J, Dembinsky-Vaknin A, Shen J, Plante M, Burt DS, Weiner HL, A nasal proteosome adjuvant activates microglia and prevents amyloid deposition, *Annals of Neurology*, 63:591-601, 2008
57. Halvorsen DS, Hansen JB, Grimsgaard S, Bonaa KH, Kierulf P, Nordoy A, The effect of highly purified eicosapentaenoic and docosahexaenoic acids on monocyte phagocytosis in man, *Lipids*, 32:935-942, 1997
58. Gorjao R, Verlengia R, Lima TM, Soriano FG, Boaventura MF, Kanunfre CC, Peres CM, Sampaio SC, Otton R, Folador A, Martins EF, Curi TC, Portiolli EP, Newsholme P, Curi R, Effect of docosahexaenoic acid-rich fish oil supplementation on human leukocyte function, *Clinical Nutrition*, 25:923-938, 2006
59. An WS, Kim HJ, Cho KH, Vaziri ND, Omega-3 fatty acid supplementation attenuates oxidative stress, inflammation, and tubulointerstitial fibrosis in the remnant kidney, *American Journal of Physiology - Renal Physiology*, 297:F895-903, 2009
60. Troseid M, Arnesen H, Hjerkinn EM, Seljeflot I, Serum levels of interleukin-18 are reduced by diet and n-3 fatty acid intervention in elderly high-risk men, *Metabolism: Clinical & Experimental*, 58:1543-1549, 2009
61. Serhan CN, Yang R, Martinod K, Kasuga K, Pillai PS, Porter TF, Oh SF, Spite M, Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions, *Journal of Experimental Medicine*, 206:15-23, 2009
62. Yagnik DR, Evans BJ, Florey O, Mason JC, Landis RC, Haskard DO, Macrophage release of transforming growth factor beta1 during resolution of monosodium urate monohydrate crystal-induced inflammation, *Arthritis & Rheumatism*, 50:2273-2280, 2004
63. Hashimoto M, Hossain S, Shimada T, Shido O, Docosahexaenoic acid-induced protective effect against impaired learning in amyloid beta-infused rats is associated with increased synaptosomal membrane fluidity, *Clinical & Experimental Pharmacology & Physiology*, 33:934-939, 2006

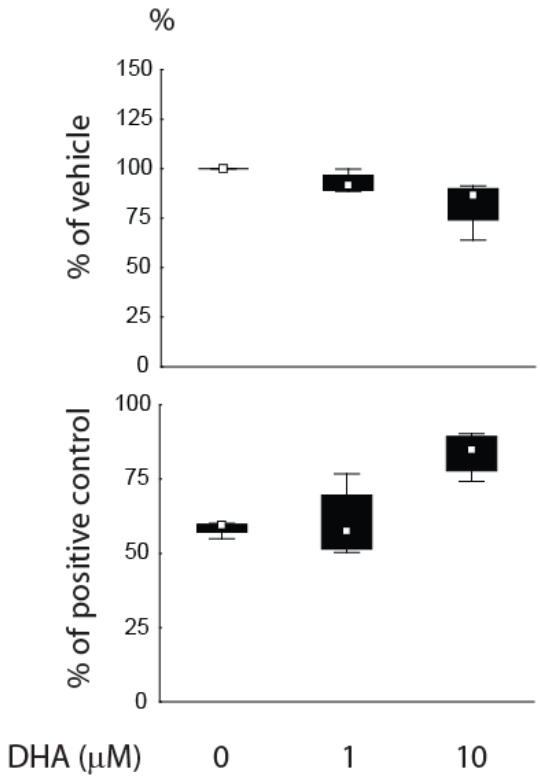

Fig. 1 Hjorth et al, 2010

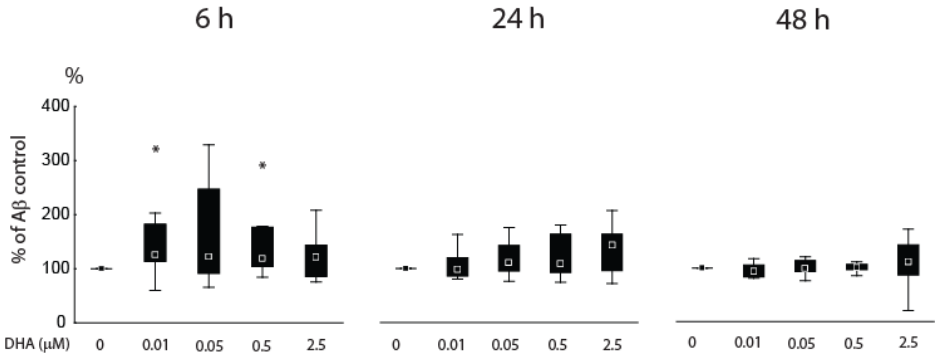

Fig. 2

Hjorth et al, 2010

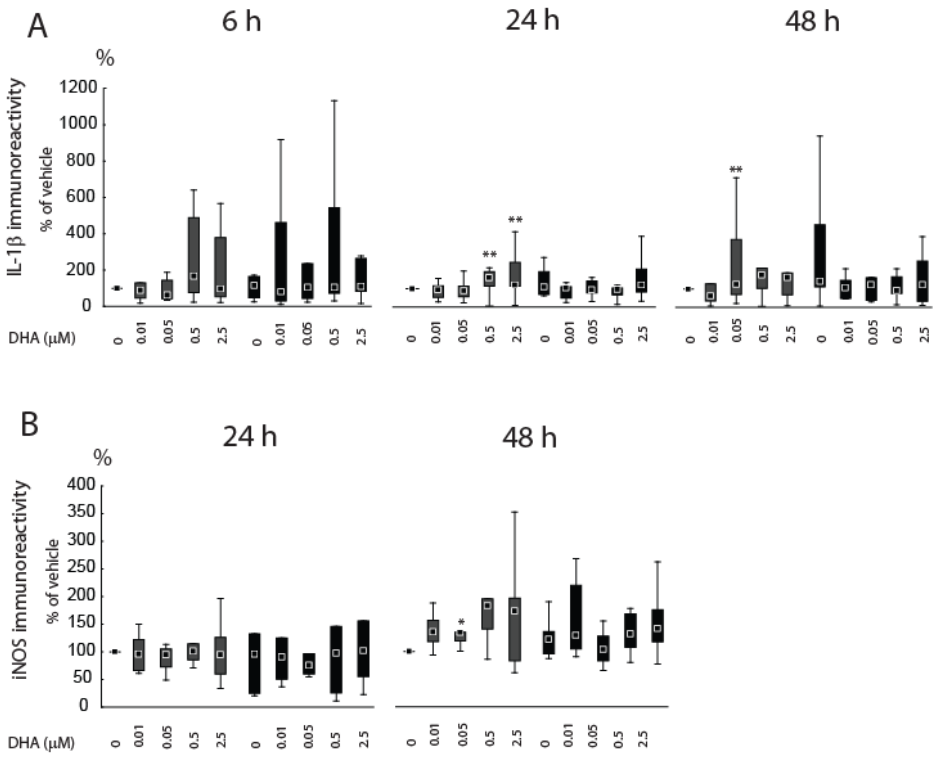

Fig. 3A-B Hjorth et al, 2010

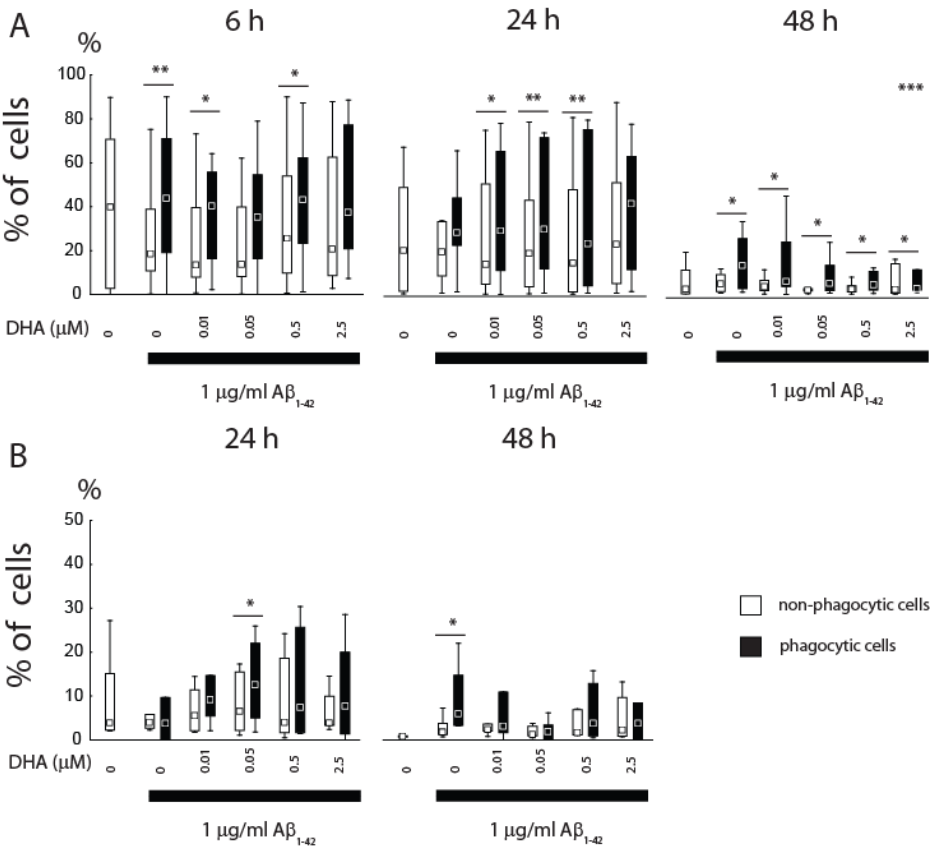

Fig. 4A-B Hjorth et al, 2010

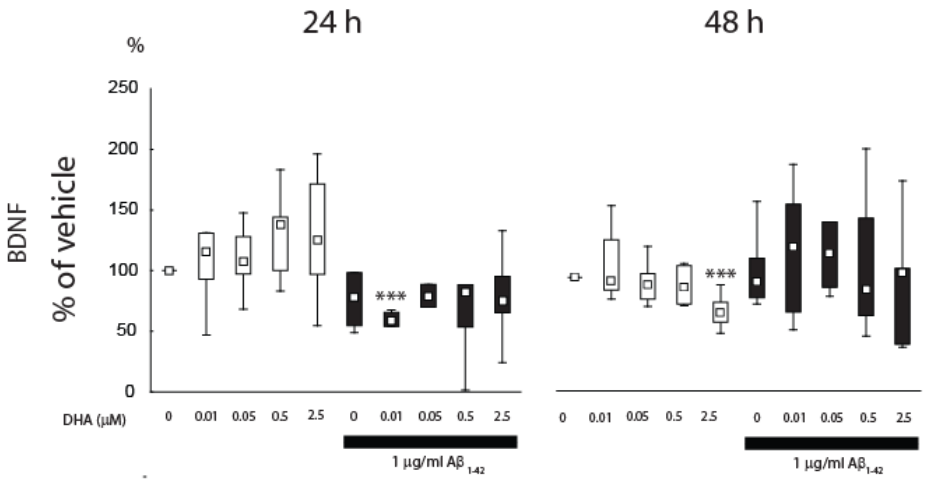

Fig. 5 Hjorth et al, 2010

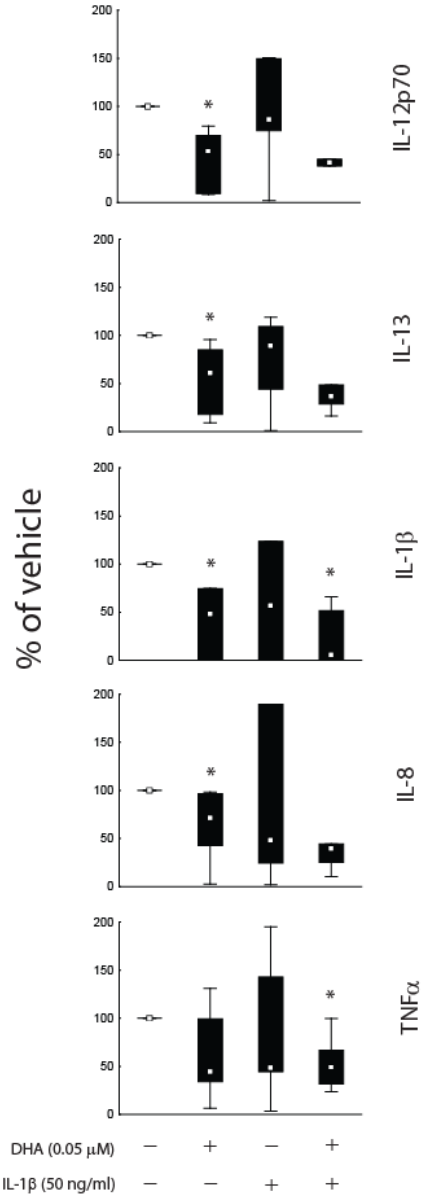

Fig. 6 Hjorth et al, 2010

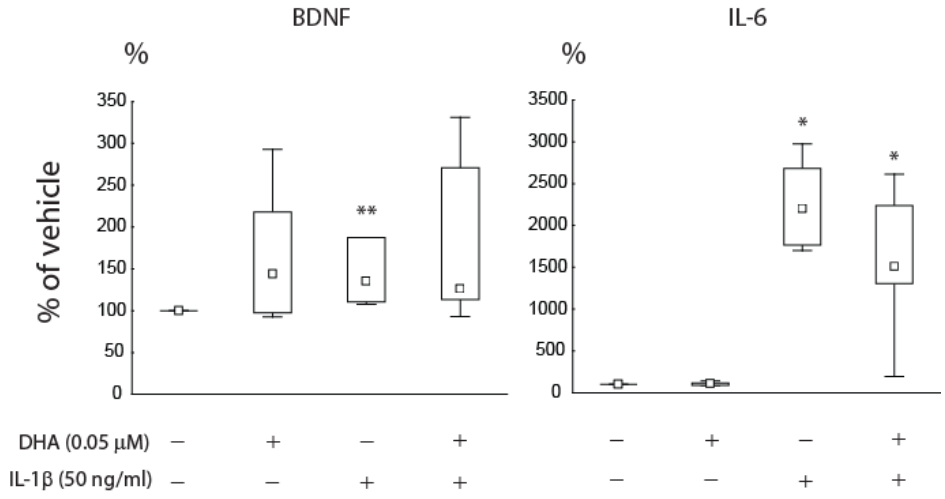

Fig. 7 Hjorth et al, 2010

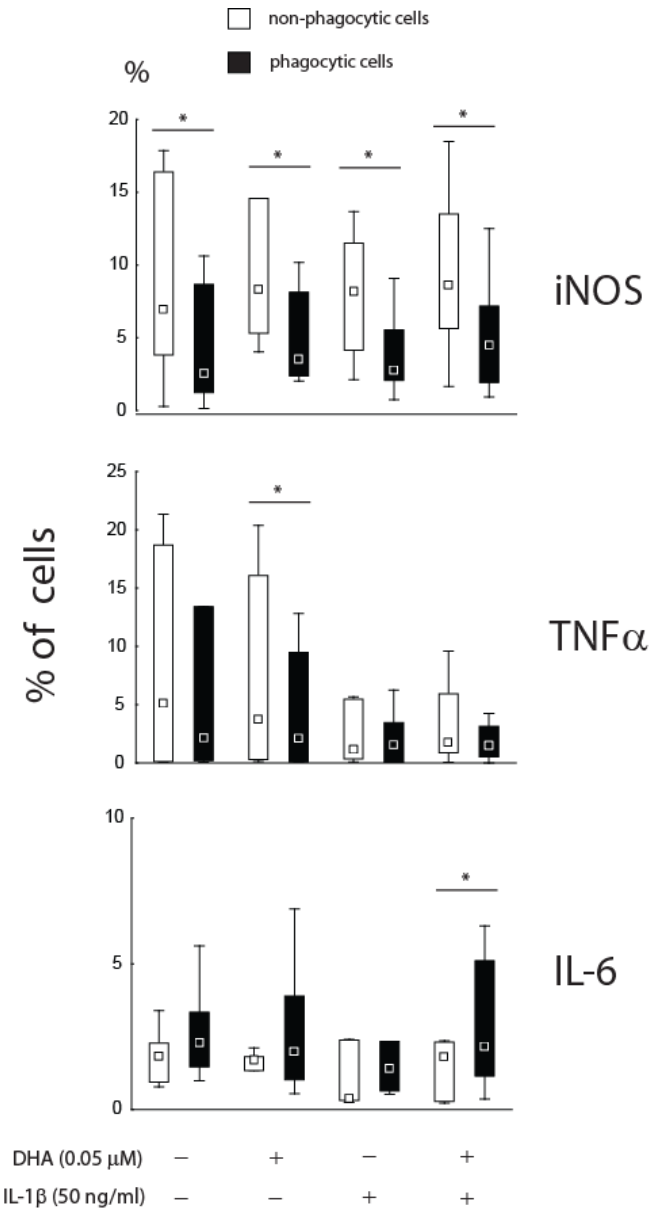

Fig. 8 Hjorth et al, 2010